GRAS Notice for *Pichia kudriavzevii* ASCUSDY21 for Use as a Direct Fed Microbial in Dairy Cattle

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GRAS Notice for *Pichia kudriavzevii* ASCUSDY21 for Use as a Direct Fed Microbial in Dairy Cattle

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LIST OF ABBREVIATIONS

AAFCO	Association of American Feed Control Officials
ADF	Acid Detergent Fiber
ANI	Average Nucleotide Identity
AOAC	Association of Official Analytical Chemists
BAM	Bacteriological Analytical Manual
BLAST	Basic Local Alignment Search Tool
BUSCO	Benchmarking Universal Single-Copy Orthologs
CFR	Code of Federal Regulations
CFU	Colony Forming Units
cGMP	current Good Manufacturing Practices
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of Variation
CVM	Center for Veterinary Medicine
DFM	Direct Fed Microbial
DM	Dry matter
DNA	DeoxyriboNucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FSMA	Food Safety Modernization Act
GC	Guanine-Cytosine
GMO	Genetically Modified Microorganism
GRAS	Generally Recognized As Safe
HACCP	Hazards Analysis Critical Control Points
ITS	Internal Transcribed Spacer
MIC	Minimum Inhibitory Concentrations
NCBI	National Center for Biotechnology Information
ND	Not Detected
NDF	Neutral Detergent Fiber
NRRL	Agricultural Research Service Culture Collection
OP	Official Publication
QPS	Qualified Presumption of Safety
RNA	RiboNucleic acid
SD	Standard Deviation
SPC	Spiral Plate Count
TMR	Total Mixed Ration
USC	United States Code
USP	United States Pharmacopoeia
YPD	Yeast extract Peptone Dextrose

NOMENCLATURE

The notified substance is *Pichia kudriavzevii* ASCUSDY21 (microbial strain) and is deposited in the NRRL culture collection as Y-67249. The microbial strain may be encapsulated with hydrogenated glycerides for use in direct fed microbial products for dairy cattle which is referred to as 'fat encapsulated *Pichia kudriavzevii* ASCUSDY21'.

The microbial strain *Pichia kudriavzevii* ASCUSDY21 is often referred to as 'Dairy-21' in some appended reports, which is the internal research name for *Pichia kudriavzevii* ASCUSDY21.

GRAS Notice for *Pichia kudriavzevii* ASCUSDY21 for Use as a Direct Fed Microbial in Dairy Cattle

PART 1 – SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR §570 Subpart E consisting of §570.203 to 280, ASCUS Biosciences, Inc. hereby informs the U.S. Food and Drug Administration (FDA) that they are submitting a Generally Recognized As Safe (GRAS) notice for *Pichia kudriavzevii* ASCUSDY21.

1.1 Name and Address of Organization

ASCUS Biosciences, Inc.
6450 Lusk Blvd Suite E209
San Diego
CA 92121

1.2 Name of the Notified Substance

The notified substance is *Pichia kudriavzevii* ASCUSDY21 (microbial strain). It is manufactured as a freeze dried milled product which is further standardized and stabilized by encapsulation in fat for use in direct fed microbial products for dairy cattle. The standardized product is referred to as 'fat encapsulated *Pichia kudriavzevii* ASCUSDY21' or '*Pichia kudriavzevii* ASCUSDY21 encapsulated'. In addition, a number of the appended reports refer to *P. kudriavzevii* ASCUSDY21 or the fat encapsulated product under the internal research name, Dairy-21.

1.3 Intended Conditions of Use

P. kudriavzevii ASCUSDY21 is intended for use as a supplemental source of viable microorganisms in the feed of dairy cattle. The intended purpose of supplementation of the live microorganism is to augment the digestion of complex carbohydrates, such as starch and cellulose. The microbial strain (notified substance) will be delivered in the fat encapsulated form to dairy cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing fat encapsulated *P. kudriavzevii* ASCUSDY21 may be incorporated into the diet of dairy cattle include as part of the total mixed ration (TMR), as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. It is anticipated that *P. kudriavzevii* ASCUSDY21 will be incorporated into feed at a recommended level of $1x10^8$ CFU/cow/day.

1.4 Statutory Basis for the Conclusion of GRAS Status

Pursuant to 21 CFR §570.30(a) and (b), *P. kudriavzevii* ASCUSDY21 manufactured by ASCUS Biosciences, has been concluded to have GRAS status for use as a direct fed microbial in dairy cattle, as described in Part 1.3, on the basis of scientific procedures.

1.5 Premarket Exception Status

ASCUS Biosciences hereby informs the U.S. FDA of the view that *P. kudriavzevii* ASCUSDY21 is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (FFDCA) based on ASCUS Biosciences conclusion that the notified substance is GRAS under the conditions of intended use as described in Part 1.3 above.

1.6 Availability of Information

The data and information that serve as the basis for this GRAS notification will be made available to the U.S. FDA for review and copying upon request during customary business hours at the offices of:

ASCUS Biosciences, Inc.
6450 Lusk Blvd Suite E209
San Diego
CA 92121

In addition, upon request, ASCUS Biosciences will supply the U.S. FDA with a complete copy of the data and information either in an electronic format that is accessible for the Agency's evaluation or on paper.

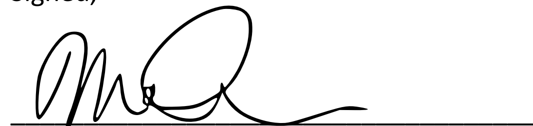
1.7 Freedom of Information Act, 5 U.S.C. 552

In ASCUS Biosciences view, all data and information presented in Parts 2 through 7 of this notice do not contain any trade secret, commercial or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempt from the Freedom of Information Act, 5 U.S.C. Section 552. A number of reports are appended to the notice, of which Appendices 011 and 015 are considered to contain proprietary commercial information which is confidential.

1.8 Certification

As required in 21 CFR 570.2225(C)(9), ASCUS Biosciences, Inc. hereby certifies that to the best of its knowledge, all data and information presented in this notice constitutes a complete, representative and balanced submission, which includes all unfavorable as well as favorable information known to ASCUS Biosciences and pertinent to the evaluation of the safety and GRAS status of *Pichia kudriavzevii* ASCUSDY21.

Signed,



18May2020

Mallory Embree, PhD, Chief Scientific Officer

Date

PART 2 – IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT

2.1 Identity

2.1.1 Taxonomic Classification

The current taxonomic classification of the *P. kudriavzevii* ASCUSDY21 is provided in Table 2.1, *Pichia kudriavzevii*, *Candida krusei*, *Issatchenkia orientalis* and *Candida glycerogenes* were recently shown by Douglass *et al*. (2018) through population genomics be the same species and in the *Pichia* genus.

Table 2.1: Taxonomic Classification of *P. kudriavzevii*

Domain	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	*Pichiaceae*
Genus	*Pichia*
Species	*kudriavzevii*

2.1.2 Source of the Microorganism

P. kudriavzevii ASCUSDY21 was identified and isolated to axenicity from a healthy, mid-lactation Holstein cow rumen sample obtained via cannula. The strain was isolated from sample DE03d9 received by ASCUS Biosciences on September 2, 2015. The isolate was deposited in the Agricultural Research Service Culture Collection (NRRL) and referenced as Y-67249. A copy of the Certificate of Deposition is provided in Appendix 001.

2.1.3 Description of the Microorganism

P. kudriavzevii ASCUSDY21 is a facultative and catalase positive yeast, forming large, creamy yellow-white colonies when cultured on solid yeast extract peptone dextrose (YPD) agar. The cells are typically large and ovoid, with terminal budding that can form strands. Images of the *P. kudriavzevii* ASCUSDY21 colonies and cells are presented in Figures 2.1 and 2.2, respectively.

Figure 2.1: *P. kudriavzevii* ASCUSDY21 Colonies on YPD Agar (Magnification 1x)

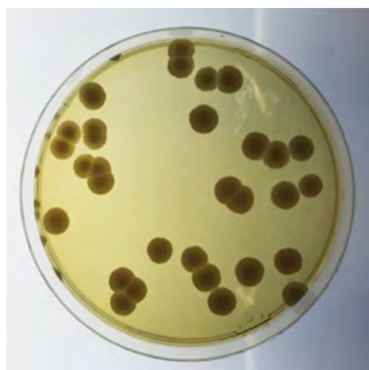
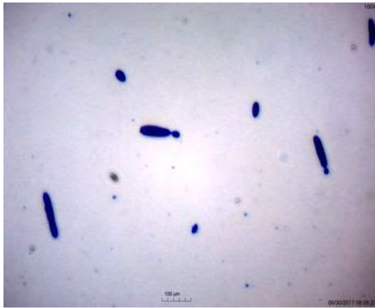


Figure 2.2: ***P. kudriavzevii* ASCUSDY21 Cells (Magnification 1000x)**



In vitro assays demonstrate that *P. kudriavzevii* ASCUSDY21 grows on a variety of soluble and insoluble carbon sources and the results are summarized in Table 2.2. *P. kudriavzevii* ASCUSDY21 utilizes various carbon sources such as glucose, fructose, and glycerol, in addition to more complex carbohydrates such as starch and cellulose, and certain organic acids as shown and summarized in Appendix 002. Similar phenotypes are reported for other *P. kudriavzevii* strains in the published literature (Yuangsaard *et al*., 2013; Arora *et al*., 2015).

Table 2.2: **Growth of *P. kudriavzevii* ASCUSDY21 on Different Carbon Sources**

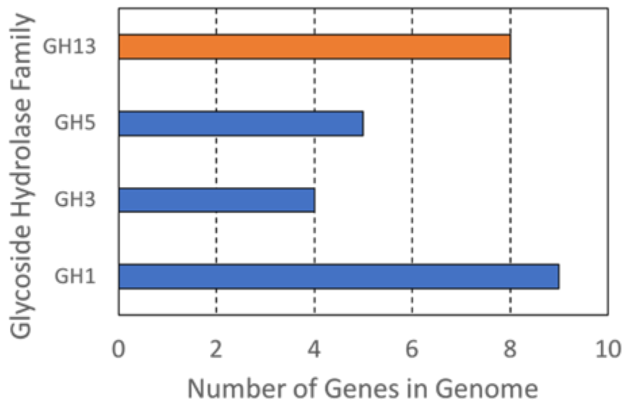
Carbon Source	Growth	Carbon Source	Growth
Cellulose	+	Lactose	-
Grass	-	Maltose	-
Bark	+	Sucrose	+
Reed Canary Grass	+	Cane molasses	+
Corn Stover	-	Beet molasses	+
Starch	+	Fructose	+
Glucose	+	Lactate	+
TMR	-	Succinate	-
Gluconate	-	Glycerol	+
Xylose	-	Arabinose	-
Mannose	+	Ribose	-
Pectin	+	Mannitol	-
Molasses	+	Sorbitol	-
Cellobiose	+	No carbon	-

Abbreviations: TMR = total mixed ration. "+" = Growth, "-" = No Growth

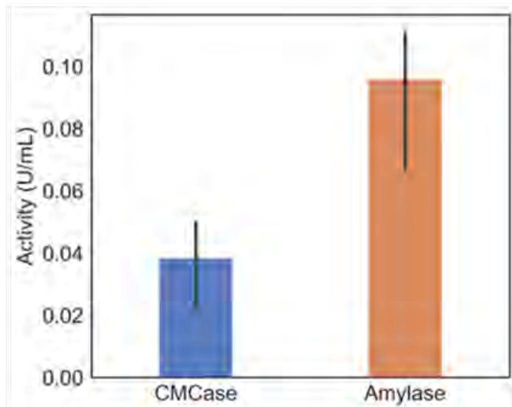
The ability of *P. kudriavzevii* ASCUSDY21 to ferment carbohydrates also was investigated using genomic analysis and *in vitro* enzymatic assays. The study report is provided in Appendix 002 and the results are summarized in Figure 2.3. The genome was identified to contain the genes necessary for degradation of starch (GH13) and cellulose (GH1, GH3 and GH5) (see Figure 2.3A). Corresponding to these findings, amylase and carboxymethylcellulose activities were displayed under the conditions of the *in vitro* assays (see Figure 2.3B).

Figure 2.3: Genes and Activities Involved in Complex Carbohydrate Fermentation by *P. kudriavzevii* ASCUSDY21

A. Carbohydrate Active Enzymes



B. Carbohydrate-Degrading Activity



Activity: Units (U)/mL of supernatant, where 1 U is the amount of enzyme required to release 1 µmol of reducing sugar/minute.

2.1.4 Identification of the Microorganism

2.1.4.1 Ribosomal DNA Marker Analysis

P. kudriavzevii ASCUSDY21 was classified as *Pichia kudriavzevii* using ribosomal DNA marker analysis. The ITS 1 and 2 sequences were amplified from *P. kudriavzevii* ASCUSDY21 using the ITS1F and ITS4 primers (Manter & Vivanco, 2007) and sequenced using an Illumina Miseq. The resulting sequence was quality trimmed and compared to National Center for Biotechnology Information (NCBI) databases using the Basic Local Alignment Search Tool (BLAST) to establish the identity of the strain. Details of the analysis are provided in Appendix 003. The results indicated that *P. kudriavzevii* ASCUSDY21 is most closely related (100%) to *Pichia kudriavzevii*.

2.1.4.2 *Whole Genome Sequence Analysis and Annotation*

Genomic DNA was isolated from a pure culture of *P. kudriavzevii* ASCUSDY21 via bead-based lysis using the MoBio PowerViral DNA kit (Carlsbad, CA). Sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA), and the resulting libraries were paired-end sequenced (2x300bp) on an Illumina Miseq. The genome was assembled using SPAdes [version 3.6.2] (Bankevich *et al*., 2012). The genome was assembled into 975 contigs and contains approximately 12,526,740 bp with a GC content of 38.11% (Table 2.3). The N50 is 37,466 bp, and the longest contig is 163,182 bp. The open reading frames were predicted through AUGUSTUS using all deposited mRNA sequences for *P. kudriavzevii* in NCBI for training (Stanke & Morgenstern, 2005).

Predicted genes were annotated through the Pfam (Finn *et al*., 2015) database and the UniRef50 Database (Suzek *et al*., 2014) using DIAMOND (Buchfink *et al*., 2015). Annotated genes were compared for universal ontology through the Gene Ontology Consortium (Gene Ontology Consortium, 2015) and mapped to functional pathways. A total of 5,807 protein-coding genes were predicted with approximately 1,107 total genes lacking a Pfam annotation and 85 lacking a match in the UniRef50 database, closely mirroring results previously found with similar genome analysis performed on a published *P. kudriavzevii* genome (Chan *et al*., 2012). The predicted set of genes is >90% complete according to a Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis (Suzek *et al*., 2014; Simão *et al*., 2015), which matches other published *P. kudriavzevii* genomes (Chan *et al*., 2012).

The assembled genome has been deposited at NCBI under accession number JABFNE000000000.

Table 2.3: *P. kudriavzevii* ASCUSDY21 Genome Assembly Metrics

# of Contigs	# of Contigs >=5,000 bp	Longest Contig (bp)	Assembly Length (bp)	N50 (bp)	N75 (bp)	GC%
975	437	163,182	12,526,740	37,466	14,189	38.11

2.1.4.3 *Whole Genome Sequence Comparison*

To determine the relatedness of *P. kudriavzevii* ASCUSDY21 to other *Pichia kudriavzevii* strains at the whole genome level, the average nucleotide identity (ANI) of the *P. kudriavzevii* ASCUSDY21 genome was compared to genomes of different *P. kudriavzevii* strains. MUMmer was used to generate the alignments for ANI on the basis that this software is good at aligning highly similar sequences and is more stringent than most other aligners such as BLAST (Kurtz *et al*., 2004). Consistent with the ITS sequence comparison, the whole genome average nucleotide identity confirmed *P. kudriavzevii* ASCUSDY21 is closely related (99%) to *Pichia kudriavzevii* strains. Full details of the analysis are provided in Appendix 003.

Table 2.4: Whole Genome ANI By MUMMER

Organism (GenBank Accession #)	Identity (%)	Coverage (%)
Pichia kudriavzevii CBS5147 (GCA_003054405)	99	98
Pichia kudriavzevii CBS573 (GCA_003054445)	99	98
Pichia kudriavzevii SJP (GCA_003033855)	99	97

2.1.4.4 Section Summary

Both 16S rRNA analysis and whole genome sequence ANI analysis have unambiguously confirmed the taxonomic identity of this commensal rumen microorganism to be *P. kudriavzevii.*

2.1.5 Plasmid Analysis

The genome assembly was assessed for plasmids through analysis of the de novo assembly graphs (Rozov *et al*., 2017). The results of the analysis are provided in Appendix 007. No horizontally acquired plasmids are detected in the *P. kudriavzevii* ASCUSDY21 genome. Analysis of the *P. kudriavzevii* ASCUSDY21 assembly graph suggests that the organism does not harbor any horizontally acquired plasmids.

2.1.6 *In-vitro* and *In-silico* Analysis of Antimicrobial Susceptibility

Phenotypic testing was conducted on *P. kudriavzevii* ASCUSDY21 to determine the minimum inhibitory concentrations (MICs) against a selected group of antifungals of relevance to human and veterinary medicine. The full study report is provided in Appendix 004.

The microbiological cut-off values were those reported by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) for fungi. This susceptibility profile is also consistent with the wild type based on previous studies on isolates (Pfaller & Diekema, 2012; Morris *et al*., 2018) and is supported by the data for *P. kudriavzevii* ASCUSDY21 in Table 2.5. The MICs obtained for *P. kudriavzevii* ASCUSDY21 were compared with available ECOFF values and breakpoints, noting the limitations of these as described below. CLSI (2018) provides clinical breakpoint tables for some *Candida* species with interpretation. For those antifungals for which breakpoints are available *P. kudriavzevii* ASCUSDY21 is considered susceptible to the echinocandin drugs anidulafungin and micafungin, and to voriconazole. Epidemiological cut-off values (ECOFFs) represent the MIC value that separates microbial populations into those with and without acquired or mutational resistance based on their phenotypes, allowing assignment of an isolate as wild-type or non-wild-type. *P. kudriavzevii* ASCUSDY21 is wild type for anidulafungin according to CLSI, and for amphotericin B according to CLSI but not EUCAST (EUCAST, 2019; CLSI, 2018). *P. kudriavzevii* ASCUSDY21 is wild type for posaconazole and itraconazole according to CLSI, but EUCAST asserts that there is insufficient evidence that these drugs are effective against *Candida krusei* (*P. kudriavzevii*) and therefore this does not allow interpretation in terms of susceptible or resistant. Wild type *Candida krusei* is known to have innate resistance to fluconazole so the MIC for this antifungal is not informative (CLSI, 2018), however, *P. kudriavzevii* ASCUSDY21 showed as susceptible for fluconazole according to EUCAST and CLSI. Morris et al. (2018) found 91.7% of *P. kudriavzevii* isolates in New Zealand were susceptible to voriconazole and 66.7% susceptible to caspofungin, although for the latter 31.1% were intermediate, with only just over 2% of *P. kudriavzevii* resistant to either antifungal. This result is consistent with the data for *P. kudriavzevii* ASCUSDY21 supporting its classification as wild type according to the data in Table 2.5.

Table 2.5:	**MIC Values *P. kudriavzevii* ASCUSDY21 (Appendix 004)**			
Antimicrobial	**Tested Range (ug/mL)**	**MIC of *P. kudriavzevii* ASCUSDY21 (ug/mL)**	**EUCAST ECOFF Values (ug/mL)**	**CLSI Non-Susceptible or Resistant (ug/mL)**
Anidulafungin	0.015-8	0.12	0.06	>2
Amphotericin B	0.12-8	2	1	>2[a]
Micafungin	0.008-8	0.25	0.25	>2
Caspofungin	0.008-8	0.5	Not Available	>2
5-Flucytosine	0.06-64	16	Not Available	≥ 32
Posaconazole	0.008-8	0.5	Not Available	Not Available
Voriconazole	0.008-8	0.5	1	≥ 4
Itraconazole	0.015-16	0.5	1	≥ 1
Fluconazole	0.12-128	64	128	≥ 64

[a] CLSI was updated to "> 2" in 2018 after completion of report.

To evaluate the presence of antimicrobial and antifungal resistance genes in the *P. kudriavzevii* ASCUSDY21 genome, all predicted genes from Section 2.1.4.2 were aligned to the comprehensive antibiotic resistance database (CARD) (Alcock *et al.,* 2020) and mycology antifungal resistance database (MARDy) (Nash *et al.,* 2018). MARDy, in particular, is the only fungal database for resistance genes, and is the current gold standard for fungal antimicrobial analysis (Nash *et al.,* 2018). The characteristics of both databases are described in Table 2.6. The alignment process compares *P. kudriavzevii* ASCUSDY21 genes against all known antimicrobial-resistance related genes that have been identified across the Bacterial and Fungal kingdoms. To ensure no hits are missed due to codon bias or sequencing error, protein alignments are considered a hit if they have greater than 80% identity over more than 70% query coverage. The CARD database contains 4,559 antimicrobial genes, none of which are from fungal genomes. No significant alignments were found between *P. kudriavzevii* ASCUSDY21 and the CARD database. MARDy contains 36 genes across 27 different fungal species, including the FSK1 gene found in *Candida krusei*. FKS1 is a component of glucan synthase, an enzyme involved in making polymers used during cell wall synthesis and is a common target of antifungals. While the *P. kudriavzevii* ASCUSDY21 genome contained a homologue to the FSK1 gene, it lacked the required mutations necessary for echanocandin resistance suggesting that this strain is sensitive to echanocandins (Desnos-Ollivier *et al*., 2008). Full details and results of the genomic analysis for antimicrobial and antifungal resistance can be found in Appendix 006.

Table 2.6:	**Characteristics of Databases Used to Assess Antimicrobial Resistance**				
Database Name	**Fungus Specific?**	**Number of Genes**	**Number of Fungal Genes**	**Number of *Candida* genes**	**Number of *P. kudriavzevii* entries in database**
CARD	No	4,559	0	0	0
MARDy	Yes	36	36 (100%)	1 gene (FKS1)	1 gene (FKS1)

2.1.6.1 Section Summary

MIC testing for *P. kudriavzevii* ASCUSDY21 reported values below or at established EUCAST and/or CLSI cutoffs. Genomic analysis indicates that *P. kudriavzevii* ASCUSDY21 is not resistant to clinically relevant

antimicrobial or antimycotic compounds. Together, these analyses suggest that should *P. kudriavzevii* ASCUSDY21 cause an opportunistic infection in a human or animal, it can be readily treated using standard antibiotics.

2.1.7 Antimicrobial Production

P. kudriavzevii ASCUSDY21 supernatant obtained post fermentation was tested for inhibitory activity against reference strains known to be susceptible to a range of antibiotics. No zones of inhibition were observed indicating that the strain is not an antimicrobial producer. Further details of the study are provided in Appendix 005.

2.1.8 Toxigenicity and Pathogenicity

To evaluate the presence of virulent and pathogenic genes in the *P. kudriavzevii* ASCUSDY21 genome, predicted genes from Section 2.1.4.2 were aligned to several databases. All applicable, publicly available databases were used to identify potential pathogenic genes. The characteristics of these databases are described in Table 2.7. The Victors virulence factors database (Sayers *et al*., 2018) is comprised of 5,304 genes, of which 364 are from fungi, 129 are from *Candida*, and none are from *P. kudriavzevii*. To broaden our analysis, additional fungi-specific databases were also included. The fungi specific database of fungal virulence factors (DFVF) (Lu *et al.,* 2012) is comprised of 2,058 genes, of which 2,058 are from fungi, 363 are from *Candida*, and one is from *P. kudriavzevii*. The pathogen-host interaction database (Phi-BASE) (Urban *et al.,* 2019) is comprised of 6,780 genes, of which 2,320 are from fungi, 648 are from *Candida,* and none are from *P. kudriavzevii*. In total, these databases encompass a total of 14,142 genes, of which 4,742 are from fungal species with 1,140 originating from *Candida*. Complete results of this analysis can be found in Appendix 006.

Table 2.7: Characteristics of Databases Used to Assess Pathogenicity and Virulence

Database Name	**Fungus Specific?**	**Number of Genes**	**Number of Fungal Genes**	**Number of *Candida* genes**	**Number of *P. kudriavzevii* entries in database**
Victors	No	5,304	364 (7%)	129 (2%)	No
DFVF	Yes	2,058	2,058 (100%)	363 (18%)	1 gene (Glucan Synthase)
Phi-BASE	No	6,780	2,320 (34%)	648 (10%)	No

The alignment process compares all identified *P. kudriavzevii* ASCUSDY21 genes against all known pathogen-related genes that have been identified across the Bacterial and Fungal kingdoms. To ensure no hits are missed due to codon bias or sequencing error, protein alignments are considered a hit if they have greater than 80% identity over more than 70% query coverage. Three, thirteen, and seven genes produced protein alignment hits between *P. kudriavzevii* ASCUSDY21 and the Victors, DFVF, and Phi-Base databases respectively. Results can be found in Tables 2.8, 2.9. and 2.10. None of the proteins identified by alignment to the databases were directly implicated in toxin production and were most commonly associated with growth and opportunistic pathogenicity.

Although three hits were identified in Victors (Table 2.8), none were directly implicated in pathogenicity:

- The first protein match from Victors was identified as a phosphoribosylformylglycinamidine synthase from *Vibrio cholerae*, which is an enzyme involved in purine biosynthesis. Purine biosynthesis is a common pathway used to make nucleotides, and the gene is not the cause of pathogenicity in *Vibrio cholera* (Chakraborty *et al*., 2000).
- The other two hits align to STE20, a protein kinase, and peroxin-1, a peroxisome biogenesis protein in *Cryptococcus neoformans*. Peroxisomes are common organelles that tend to house oxidative enzymes, such as catalase.

Table 2.8:	**Significant alignments between the Victors virulence database and *P. kudriavzevii* ASCUSDY21**			
Query	**Subject**	**%identity**	**evalue**	**Query coverage %**
g1100.t1	gi|15640885|ref|NP_230516.1| phosphoribosylformylglycinamidine synthase [Vibrio cholerae O1 biovar El Tor str. N16961]	80.3	2.10E-166	98.90%
g3993.t1	gi|25573205|gb|AAN75173.1| STE20 [Cryptococcus neoformans var. grubii]	82.5	1.30E-161	99.68%
g3246.t1	gi|799325851|ref|XP_012048907.1| peroxin-1 [Cryptococcus neoformans var. grubii H99]	81.5	2.00E-91	98.48%

Of the 13 matches to the DFVF, 11 were unique matches and, with 2 were redundant alignments (Table 2.9). All genes producing significant alignments fall under categories such as kinase/phosphatase activity, structural binding through actin or tubulin, and histones. with Nno genes were directly related to toxin production or virulence:

- The strongest match was to g3635.t1, which is the FKS1 gene identified by comparison to MARDy in Section 2.1.5.
- A homologue to HSP90 was identified, this is a molecular chaperone that allows for proper protein folding and is commonly found in prokaryotes and eukaryotes alike (Schopf *et al*., 2017). In pathogenic *Candida* this gene allows the organism to adapt to host physiological conditions and stabilizes molecules involved in signal transduction (O'Meara *et al*., 2017). While HSP90 is found in pathogens, it is not directly responsible for pathogenicity or virulence and is ubiquitously found in eukaryotes including humans.
- The next two hits identified were to common cytoskeleton components, actin and tubulin. While these proteins are found in pathogenic fungi they are also found in non-pathogenic yeast such as *Saccharomyces cerevisiae* and are not solely responsible for pathogenicity or virulence (Kopecka *et al*., 2001).
- A non-specific protein kinase was the next hit. Protein kinases comprise ~2% of the total genes in yeast and humans and act to facilitate signal transduction (Hunter and Plowman, 1997). While protein kinases have been shown to facilitate pathogenicity in *Candida,* they do not directly cause pathogenicity or virulence (Lin *et al*., 2018).
- A HOG1 kinase homologue was also hit. The HOG1 kinase is found in many yeasts including *S. cerevisiae* and acts to regulate pathways implicated in osmotic stress amongst others (Kim *et al*., 2016). HOG1 does act in signaling pathways in pathogenic *Candida* but does not cause virulence independently (Alonso-Monge *et al*., 2009).

- Two non-specific pyrophosphatases and one metallophosphatase were also identified. Phosphate acquisition is critical for all organisms, not only pathogenic fungal species. While it is essential for fungal pathogens to acquire phosphate during infection, it does not cause virulence or pathogenicity (Ikeh *et al.*, 2017).
- A phosphokinase-like protein generated a hit. Phosphokinases are found in both pathogenic and nonpathogenic fungi and are not known to cause pathogenicity or virulence. However, they have been investigated as drug targets due to conservation (Santini *et al.*, 2008).
- A signaling molecule in the 14-3-3 family was the next identified protein. These proteins are highly conserved in yeasts as well as other eukaryotes and interact with phosphorylated proteins (Van Heusden and Steensma, 2006). 14-3-3 family proteins are critical for growth in *Candida* but do not directly cause virulence (Cognetti *et al.*, 2002).
- The final two protein hits to DFVF were to histones. Histones are ubiquitous DNA packaging proteins. Histone modification has been implicated in pathogenicity by acting as a transcriptional regulator of genes involved in pathogenicity in *Candida* but not the histone protein itself (Lopes da Rosa *et al.*, 2009; Hnisz *et al.*, 2012).

Table 2.9: Significant alignments between the Database of Fungal Virulence Factors and *P. kudriavzevii* ASCUSDY21

Query	Subject (UniprotID|Organism|Uniprot description|Pfam description|disease)	%identity	evalue	Query coverage %
g3635.t1	Q4U2W2_ISSOR|Issatchenkia orientalis|Unknown|Glucan synthase|infection	99.5	0.00E+00	99.95%
g3017.t1	HSP90_CANAL|Candida albicans|Molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction. Undergoes a functional cycle that is linked to its ATPase activity. This cycle probably induces conformational changes in the client proteins, thereby causing their activation. Interacts dynamically with various co-chaperones that modulate its substrate recognition, ATPase cycle and chaperone function (By similarity).|HATPase_c|invasive candidal disease	84.1	3.30E-302	99.71%
g141.t1	B9WMS9_CANDC|Candida dubliniensis|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity). |Tubulin| leptomeningeal disease,occasional invasive candidal disease	81	1.60E-222	99.33%
g1478.t1	Q5A415_CANAL|Candida albicans|Belongs to the actin family.|Actin|invasive candidal disease	87	1.90E-187	92.27%

Table continued on next page.

Table 2.9: Significant alignments between the Database of Fungal Virulence Factors and *P. kudriavzevii* ASCUSDY21 (cont'd)				
Query	**Subject (UniprotID|Organism|Uniprot description|Pfam description|disease)**	**%identity**	**evalue**	**Query coverage %**
g3272.t1	Q59Z23_CANAL|Candida albicans|ATP + a protein = ADP + a phosphoprotein.|Pkinase|invasive candidal disease	89.1	3.90E-180	86.35%
g2584.t1	HOG1_CRYPA|Cryphonectria parasitica|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression.|Pkinase|Chestnut blight. Cankers	82	8.20E-178	88.89%
g1503.t1	A7ULH9_CANGY|Candida glycerinogenes|Unknown|Pyrophosphatase|Occasional invasive candidal disease	100	4.20E-171	99.65%
g2376.t1	A7ULH9_CANGY|Candida glycerinogenes|Unknown|Pyrophosphatase|Occasional invasive candidal disease	100	4.20E-171	99.65%
g3993.t1	Q59KY8_CANAL|Candida albicans|A phosphoprotein + H(2)O = a protein + phosphate.|Metallophos|invasive candidal disease	82.5	1.90E-161	99.68%
g3120.t1	C4YJK4_CANAW|Candida albicans|ATP + D-ribose 5-phosphate = AMP + 5-phospho- alpha-D-ribose 1-diphosphate.|Pribosyltran|invasive candidal disease	85	1.10E-154	99.38%
g2712.t1	1433_CANAL|Candida albicans|Belongs to the 14-3-3 family.|14-3-3|invasive candidal disease	84.1	2.70E-113	98.02%
g1267.t1	D2JLR3_9HYPO|Fusarium sp|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone|mycotoxins	94.7	5.20E-63	95.59%
g3389.t1	D2JLR3_9HYPO|Fusarium sp|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone|mycotoxins	94.7	5.20E-63	95.59%

The 7 genes from *P. kudriavzevii* ASCUSDY21 that aligned to the Phi-Base database were associated with "reduced_virulence" or "loss_of_pathogenicity" (Table 2.10). This database links genomic mutants to phenotypic response, so these entries should be read as gene deletions that result in reduced virulence or loss of pathogenicity. Therefore, the hits identified by Phi-Base are genes observed in non-pathogenic strains of *P. kudriavzevii*.

Table 2.10:	**Significant alignments between the Pathogen-Host Interactions Database and *P. kudriavzevii* ASCUSDY21**			
Query	**Subject**	**%identity**	**evalue**	**Query coverage %**
g2584.t1	Q6FIU2 PHI:4625_PHI:8395 CgHog1__Cghog1 5478 Candida_glabrata reduced_virulence_unaffected_ pathogenicity	82.3	1.20E-183	91.21%
g3272.t1	C5M7A4 PHI:7811 CaTpk2_(CTRG_01736) 5482 Candida_tropicalis loss_of_pathogenicity__ unaffected_pathogenicity	88.8	5.10E-179	86.35%
g1100.t1	P04173 PHI:504 LEU2 4932 Saccharomyces_cerevisiae reduced_virulence	80.3	1.10E-165	98.90%
g3993.t1	Q59KY8 PHI:378 SIT4 5476 Candida_albicans reduced_virulence	82.5	6.80E-161	99.68%
g2509.t1	Q5A5Q8 PHI:6076 RPS41 5476 Candida_albicans reduced_virulence	83.2	6.30E-122	99.59%
g402.t1	Q5AND9 PHI:7009 arf2 5476 Candida_albicans reduced_virulence	89.5	3.30E-91	99.45%
g3246.t1	O42825 PHI:270 RHO1 5476 Candida_albicans loss_of_pathogenicity	81.5	1.00E-90	98.48%

For comparative purposes, and to better gauge the global pool of virulence factors in *P. kudriavzevii* and related species, amino acid sequences for published genomes of *P. kudriavzevii* (accession #GCA_003054445) (Douglass *et al.*, 2018) and *P. kudriavzevii* (accession # GCA_002166775) (Cuomo *et al.*, 2017) were downloaded from NCBI Genbank and analyzed using the same method as *P. kudriavzevii* ASCUSDY21. Both of these isolates were previously published and deposited as *Candida krusei* before the revision of nomenclature to *P. kudriavzevii*. Additionally, the amino acid sequences for the closely related *Pichia membranifaciens* (accession # GCA_001661235) (Riley *et al.*, 2016) was downloaded for comparison.

The results of the alignment are presented in Table 2.11. Victors returned 4, 4, and 6 hits to *P. kudriavzevii / C. krusei* (GCA_003054445), *P. kudriavzevii / C. krusei* (GCA_002166775), and *P. membranifacien*s (GCA_001661235) respectively.

DFVF returned 68, 66, and 86 hits to *P. kudriavzevii / C. krusei* (GCA_003054445), *P. kudriavzevii / C. krusei* (GCA_002166775), and *P. membranifaciens* (GCA_001661235) respectively. After removing redundant hits to subject and query sequences in the DFVF, hits to the DFVF database numbered 16, 13, and 18 to *P. kudriavzevii / C. krusei* (GCA_003054445), *P. kudriavzevii / C. krusei* (GCA_002166775), and *P.* membranifaciens (GCA_001661235) respectively.

Hits to PhiBase numbered 25, 23, and 46 to *P. kudriavzevii / C. krusei* (GCA_003054445), *P. kudriavzevii / C. krusei* (GCA_002166775), and *P. membranifaciens* (GCA_001661235) respectively. After removing redundant hits to subject and query sequences in the PhiBase, hits to the PhiBase database numbered 12, 10, and 16 to *P. kudriavzevii / C. krusei* (GCA_003054445), *P. kudriavzevii / C. krusei* (GCA_002166775), and *P.* membranifaciens (GCA_001661235) respectively.

Similarly to *P. kudriavzevii* ASCUSDY21, all significant alignments fall under the category of growth and opportunistic pathogenicity, and do not directly cause disease. No genes directly related to

pathogenesis or toxin production were identified in the downloaded genomes. A table containing a list of the hits to the databases are located in Tables 1-3 in Appendix 006.

Table 2.11: **Number of Alignments Between Pathogenicity and Virulence Databases and Publicly Available Genomes of Organisms Closely Related to *P. kudriavzevii* ASCUSDY21**			
Genome	**Victors**	**DFVF**	**PhiBase**
P. kudriavzevii / *C. krusei* (GCA_003054445)	4	68 / 16 (nonredundant)	25 / 12 (nonredundant)
P. kudriavzevii / *C. krusei* (GCA_002166775)	4	66 / 13 (nonredundant)	23 / 10 (nonredundant)
P. membranifaciens (GCA_001661235)	6	86 / 18 (nonredundant)	46 /16 (nonredundant)

While the downloaded genomes provided more hits to the databases than *P. kudriavzevii* ASCUSDY21, the numbers are inflated. Many of the sequences producing significant alignments to the DFVF and Phi-Base databases align to the same query sequence from the downloaded genomes. Furthermore, subject sequences which provide hits to the same query sequences in the downloaded genomes have the same functional annotation and alignment identity. The only difference between these hits are that the subject proteins originate from different species and were thus given different IDs in the respective databases. For example, query sequence AWU76275.1 in *P. kudriavzevii / C. krusei* (GCA_003054445) aligned to 9 different subject sequences in the DFVF database. All 9 of the subject sequences matched query sequence AWU76275.1 at 94.66% and were annotated as histones. The only difference between the subject proteins was that they were derived from different fungal species. These proteins should be considered homologous and effectively redundant entries. If redundant hits to the same region in the *P. kudriavzevii* ASCUSDY21 genome are condensed the resulting hits to the DFVF database number 16, 13, and 18 and Phi-Base hits number 12, 10, and 16 to *C. krusei / P. kudriavzevii* (GCA_003054445), *C. krusei / P. kudriavzevii* (GCA_002166775), and *P. membranifaciens* (GCA_001661235) respectively. Similarly, to the genes identified in *P. kudriavzevii* ASCUSDY21, these hits are to histones, actin/tubulin, kinases, phosphatases, signal transduction molecules, and do not directly contribute to toxin production and pathogenicity. None of the hits belong to proteins considered responsible in pathogenesis such as toxins, proteases, phospholipases, superoxide dismutases, DNases, and ureases (Almeida *et al*., 2015).

2.1.8.1 Section Summary

No genes directly involved in pathogenesis or toxin production were identified.

All publicly available pathogen and virulence-related databases were queried to determine the pathogenic potential of *P. kudriavzevii* ASUCSDY21. In total, these databases encompass 14,142 known pathogen-related genes spanning all microbial taxonomies, of which 4,742 are from fungal species, and 1,140 originated from *Candida* species. Comprehensive alignment of the *P. kudriavzevii* ASCUSDY21 genome to these databases yielded 23 hits at 80% identity, 70% query coverage.

Further investigation of alignments revealed no genes directly involved in pathogenesis or toxin production. Genes that aligned to the databases were either structural or related to general cell function. No genes directly involved in pathogenesis or toxin production were identified. To better assess the completeness of the analysis, genomes of two publicly available *C. krusei / P. kudriavzevii* and close relative *P. membranifaciens* were analyzed using the same methods to assess completeness of the

analysis: 32, 27, and 40 nonredundant genes aligned for *C. krusei / P. kudriavzevii* (GCA_003054445), *C. krusei / P. kudriavzevii* (GCA_002166775), and *P. membranifaciens* (GCA_001661235) respectively. No genes directly involved in pathogenesis or toxin production were identified.

2.1.9 Genetic Stability

The genetic stability of *P. kudriavzevii* ASCUSDY21 was assessed throughout the manufacturing process. Three independent samples from the two working cell banks as well as the cell concentrate at the end of fermentation and the freeze-dried powder were analyzed for similarity to the master cell bank. Over the manufacturing process, a typical seed of 10^6 CFU must undergo around 40 generations to reach 10^{18}CFU estimated for the main fermenter. Similarity was >99.9% for all samples testing indicating minimal genetic drift during manufacturing. The results are reported in Appendix 008.

2.1.10 Summary of Organism Safety Based on Genomics

P. kudriavzevii ASCUSDY21 was unambiguously identified using 16S rRNA analysis and whole genome sequence ANI analysis. *P. kudriavzevii* ASCUSDY21 is not resistant to clinically relevant any antimicrobial or antimycotic compounds, suggesting that should *P. kudriavzevii* ASCUSDY21 cause an opportunistic infection in a human or animal, it can be readily treated using standard antibiotics. Additionally, phenotypic testing confirmed that no antimicrobials were produced during fermentation. *P. kudriavzevii* ASCUSDY21 is also genetically stable during the fermentation process. Comparison of the *P. kudriavzevii* ASCUSDY21 genome to several databases containing known pathogenic-related genes yielded 23 hits. No genes were associated with toxin production, pathogenicity, or virulence. To better assess the completeness of the analysis, genomes of two publicly available *C. krusei / P. kudriavzevii* and close relative *P. membranifaciens* were analyzed using the same methods to assess completeness of the analysis: 32, 27, and 40 nonredundant genes aligned for *C. krusei / P. kudriavzevii* (GCA_003054445), *C. krusei / P. kudriavzevii* (GCA_002166775), and *P. membranifaciens* (GCA_001661235) respectively. No genes directly involved in pathogenesis or toxin production were identified. Based on these analyses, *P. kudriavzevii* ASCUSDY21 is safe for use as direct fed microbial.

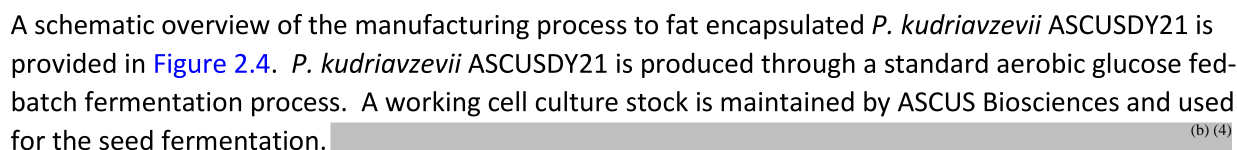
2.2 Method of Manufacture

2.2.1 Raw Materials and Processing Aids

The raw materials and processing aids used in the manufacture of fat encapsulated *P. kudriavzevii* ASCUSDY21 are listed in Appendices 009A to 009Y. All raw materials used in the manufacture of fat encapsulated *P. kudriavzevii* ASCUSDY21 have a history of use in the industrial food and feed fermentation processes, and are considered by ASCUS Biosciences to be safe and suitable for use in the manufacture of feed ingredients in the U.S.

2.2.2 Manufacturing Process

A schematic overview of the manufacturing process to fat encapsulated *P. kudriavzevii* ASCUSDY21 is provided in Figure 2.4. *P. kudriavzevii* ASCUSDY21 is produced through a standard aerobic glucose fed-batch fermentation process. A working cell culture stock is maintained by ASCUS Biosciences and used for the seed fermentation.



(b) (4) *P. kudriavzevii* ASCUSDY21 is then (b) (4). (b) (4) (b) (4) Details of the manufacturing process are provided in Appendix 011 (Confidential).

Figure 2.4: Schematic Overview of the Manufacturing Process

2.2.3 Production Controls

The commercial manufacture of fat encapsulated *P. kudriavzevii* ASCUSDY21 will be in accordance with current Good Manufacturing Practices (cGMP) and a Hazards Analysis Critical Control Points (HACCP) plan will be in place. The requirements of the Food Safety Modernization Act (FSMA) as laid down in 21 CFR §117 will be applied at all stages of the production, processing and distribution of the feed product.

2.3 Product Specifications and Batch Analyses

2.3.1 Product Specifications for Freeze Dried *P. kudriavzevii* ASCUSDY21 (Notified Substance)

Appropriate feed-grade specifications have been established for *P. kudriavzevii* ASCUSDY21 manufactured as a freeze dried powder and are presented in Table 2.12. Copies of the methods of analysis are provided in Appendices 012A to 012H.

Table 2.12: *P. kudriavzevii* ASCUSDY21 Product Specifications

Parameter	Specification Limits	Analytical Method
Viable cells count	(b) (4)	Internal method (see Appendix 012H)

Abbreviations: CFU = colony forming units; Internal method (Appendix 012H)

2.3.2 Batch Analyses for Freeze Dried *P. kudriavzevii* ASCUSDY21 (Notified Substance)

Three batches of *P. kudriavzevii* ASCUSDY21 (freeze dried cell concentrate; notified substance) representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed specifications. The results are summarized in Table 2.13 and the Certificates of Analysis are provided in Appendix 013.

Table 2.13: Analytical Results for 3 Batches of *P. kudriavzevii* ASCUSDY21

			Analytical Results		
Parameter	Unit	Specification	Lot 18-0202-001-P84-1	Lot 18-0202-001-P85-1	Lot 18-0202-001-P85-2
Viable cells count	CFU/g	(b) (4)	(b) (4)		

Abbreviations: CFU = colony forming units.

2.3.3 Product Specifications for the Fat Encapsulated Product

Appropriate feed-grade specifications have been established for fat encapsulated *P. kudriavzevii* ASCUSDY21 and are presented in Table 2.14. Copies of the methods of analysis are provided in Appendices 012A to 012H.

Table 2.14: Fat Encapsulated *P. kudriavzevii* ASCUSDY21 Product Specifications

Parameter	Specification Limits	Analytical Method
Viable cells count	(b) (4)	Internal method (see Appendix 012A)
Coliforms		BAM-SPC
E. coli		BAM-SPC
Salmonella		AOAC 2013.01
Listeria		AOAC 2013.10

Abbreviations: CFU = colony forming units; BAM-SPC = Bacteriological Analytical Manual – Spiral Plate Count; AOAC = Association of Official Analytical Chemists, Internal method (Appendix 012A)

2.3.4 Batch Analyses for Fat Encapsulated Product

Three batches of fat encapsulated *P. kudriavzevii* ASCUSDY21 representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed product specifications. The results are summarized in Table 2.15 and the Certificates of Analysis are provided in Appendix 014.

Table 2.15: Analytical Results for 3 Batches of Fat Encapsulated *P. kudriavzevii* ASCUSDY21

Parameter	Unit	Specification	Analytical Results		
			Lot 18-0202-001-P86-1	Lot 18-0202-001-P86-2	Lot 18-0202-001-P87-1
Viable cells count	CFU/g				(b) (4)
Coliforms	CFU/g				
E. coli	Per 25 g				
Salmonella	Per 25 g				
Listeria	Per 25 g				

Abbreviations: CFU = colony forming units.

2.3.5 Additional Analytical Data

The levels of heavy metals are also routinely monitored in fat encapsulated *P. kudriavzevii* ASCUSDY21. Three batches of fat encapsulated *P. kudriavzevii* ASCUSDY21 representative of the commercial material were analyzed to verify that the levels of these contaminants fall within acceptable ranges. The results are summarized in Table 2.16 and the Certificates of Analysis provided in Appendix 014. On the basis of the analytical data, no specifications for heavy metals are required.

Table 2.16: Further Analytical Results for 3 Batches of Fat Encapsulated *P. kudriavzevii* ASCUSDY21

Parameter	Unit	Analytical Results			Analytical Method
		Lot 18-0202-001-P86-1	Lot 18-0202-001-P86-2	Lot 18-0202-001-P87-1	
Arsenic	ppm			(b) (4)	AOAC 2015.01
Cadmium	ppm				AOAC 2015.01
Lead	ppm				AOAC 2015.01
Mercury	ppm				AOAC 2015.01

Abbreviations: AOAC = Association of Official Analytical Chemists; "<" refers to analytical results falling below detection limits.

2.4 Stability

2.4.1 Stability Data

ASCUS Biosciences guarantees conformity of fat encapsulated *P. kudriavzevii* ASCUSDY21 to the product specifications (see Table 2.17) for 12 months when stored in the original, unopened (sealed) packaging at temperatures of between 2 and 10°C. The proposed shelf-life is supported by ongoing stability studies in which 3 batches of fat encapsulated *P. kudriavzevii* ASCUSDY21 in packaging representative of the commercial material are stored at 5°C, 25°C and 40°C, respectively. Packaging information is

provided in Appendix 015A. The available real-time data at 5°C currently support a shelf-life of 12 months.

2.4.1.1 Stability Study at 5°C

The results of the stability study conducted at 5°C for 12 months on fat encapsulated *P. kudriavzevii* ASCUSDY21 are summarized in Table 2.17 and the report is provided in Appendix 015B, Confidential. Over the period evaluated to date, changes in the viable cell count are consistent with the proposed product specification (b) (4)) and a shelf life of 12 months at 2-10°C.

Table 2.17: Results of a Stability Study on 3 Batches of Fat Encapsulated *P. kudriavzevii* ASCUSDY21 Stored at 5°C

Time (Months)	Unit	Analytical Results					
		Lot 18-0202-001-P86-1		Lot 18-0202-041-P86-2		Lot 18-202-001-P87-1	
		Viable Cells Count	SD	Viable Cells Count	SD	Viable Cells Count	SD
0	CFU/g						(b) (4)
1	CFU/g						
2	CFU/g						
3	CFU/g						
6	CFU/g						
9	CFU/g						
12	CFU/g						

Abbreviations: CFU = colony forming units; SD = standard deviation.

2.1.4.2 Stability Study at 25°C

The results of the stability study conducted at 25°C for 12 months on fat encapsulated *P. kudriavzevii* ASCUSDY21 are summarized in Table 2.18 and the report is provided in Appendix 015C, Confidential. Over the period evaluated to date, changes in the viable cell count are consistent with the proposed product specification ((b) (4)) and a shelf life of 12 months at 2-10°C.

Table 2.18: Results of a Stability Study on 3 Batches of Fat Encapsulated *P. kudriavzevii* ASCUSDY21 Stored at 25°C

Time (Months)	Unit	Analytical Results					
		Lot 18-0202-001-P86-1		Lot 18-0202-041-P86-2		Lot 18-202-001-P87-1	
							(b) (4)
0	CFU/g						
1	CFU/g						
2	CFU/g						
3	CFU/g						
6	CFU/g						
9	CFU/g						
12	CFU/g						

Abbreviations: CFU = colony forming units; SD = standard deviation.

2.1.4.3 Stability Study at 40°C

The results of a stability study conducted at 40°C for 26 weeks on fat encapsulated *P. kudriavzevii* ASCUSDY21 are summarized in Table 2.19 and the report is provided in Appendix 015D, Confidential. Over the period evaluated to date, changes in the viable cell count are consistent with the proposed product specification ((b) (4) and a shelf life of 6 months at 2-10°C.

Table 2.19: Results of a Stability Study on 3 Batches of Fat Encapsulated *P. kudriavzevii* ASCUSDY21 Stored at 40°C

Time (Weeks)	Unit	Analytical Results					
		Lot 18-0202-001-P86-1		Lot 18-0202-041-P86-2		Lot 18-202-001-P87-1	
		Viable Cells Count	SD	Viable Cells Count	SD	Viable Cells Count	SD
0	CFU/g						(b) (4)
1	CFU/g						
2	CFU/g						
3	CFU/g						
4	CFU/g						
13	CFU/g						
26	CFU/g						

Abbreviations: CFU = colony forming units; SD = standard deviation.

2.4.2 <u>In-Feed Stability</u>

As mentioned in Part 1, fat encapsulated *P. kudriavzevii* ASCUSDY21 may be incorporated into the diet of dairy cattle as part of the TMR, as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. The strain is encapsulated using fat to generate a stable product suitable for handling under practical commercial farming conditions in the U.S. The dry matter intake of dairy cattle is optimized by feeding fresh TMR on a twice daily basis. The forage content is typically adjusted to meet the nutrient requirements of the animals on a pen basis. Under the conditions of intended use, fat encapsulated *P. kudriavzevii* ASCUSDY21 may be mixed directly into the TMR or added as a top-dressing at the point of use, at the time of feeding. On this basis, long-term stability is not relevant and an in-feed stability study was not conducted. Demonstration that *P. kudriavzevii* ASCUSDY21 survives the mixing process in feed is provided by the homogeneity study (see below, Part 2.4.3).

2.4.3 <u>Homogeneity Data</u>

The ability of fat encapsulated *P. kudriavzevii* ASCUSDY21 to be mixed homogeneously into feed was evaluated. A direct fed microbial product containing fat encapsulated *P. kudriavzevii* ASCUSDY21 was incorporated into 3 samples of grain at 5 g/lb, equivalent 1 to x10^8 CFU/lb and representative of practical conditions of use in feed. Ten sub-samples of grain were collected across the mixer and analyzed for *P. kudriavzevii* viable cells count. The coefficients of variation (CV) in viable cells count for the 10 sub-samples was determined for each grain sample and the results are summarized in Table 2.20 and Appendix 015E. The CV reported for *P. kudriavzevii* viable cells count are relatively consistent (%) among the 3 grain samples indicating that under the conditions of intended use, fat encapsulated

P. kudriavzevii ASCUSDY21 can be homogeneously distributed into feed. Background *Pichia* species present naturally in the feed will contribute to the viable cell counts and CV variability.

Table 2.20: Results of a Homogeneity Study on 3 Samples of Grain Premix Containing Fat Encapsulated *P. kudriavzevii* ASCUSDY21

Grain Sample	Analytical Data			Calculations	
	Unit	Mean Viable Cells Count	SD	Unit	CV (%)
1	CFU/g				(b) (4)
2	CFU/g				
3	CFU/g				

Abbreviations: CFU = colony forming units; SD = standard deviation; CV = coefficient of variation.

2.4.4 Manufacturing Summary

Ascus Biosciences Inc will manufacture a safe stable product for dairy cattle meeting cGMP and FSMA compliance. This was demonstrated through batches of product meeting product specifications for contaminants, heavy metals and potency. The fat encapsulation helps to improve the product stability through feeding and mixing in the normal dairy ration. The product is packaged in moisture protected barrier bags.

2.5 Effect of the Notified Substance

This portion of the notice addresses the requirements specified in 21 CFR 570.230(d):

> (d) When necessary to demonstrate safety, relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of the notified substance required to produce such effect.

The GRAS Final Rule (81 FR 54960) provides interpretation of this regulation specific to animal feed ingredients in response to comment 144: "We agree that data and information bearing on the physical or other technical effect the notified substance is intended to produce are only necessary when they bear on safety." A product like phytase would require data, however, the intended purpose of supplementation of *P. kudriavzevii* ASCUSDY21 is to augment normal rumen digestion of complex carbohydrates, such as starch and cellulose. As described below, ASCUS Biosciences has determined that the technical effect of *P. kudriavzevii* ASCUSDY21 when fed to dairy cattle as a direct fed microbial under the conditions of intended use does not have a bearing on safety. Thus, data and information demonstrating the intended effect of *P. kudriavzevii* ASCUSDY21 in the feed of dairy cattle are not required as part of this GRAS notice.

The use of this organism is to facilitate the digestion of complex carbohydrates such as starch and cellulose of animal feed within the rumen, as the enzymes of interest are related to amylase and carboxymethylcellulose. *P. kudriavzevii* has been fed to dairy cattle previously. Intanoo *et al*. (2020) fed *P. kudriavzevii* and *Kluyveromyces marxianus* to dairy cows and found that supplementation improved detoxification of aflatoxin B_1, and improved DMI and milk components. No impacts on animal health were observed. The contribution of DFMs to the fermentation characteristics of the rumen has been extensively evaluated (Elghandour, 2015), and is further described below in context of technical effect and animal safety (Section 6.4 of this notice).

2.5.1 Rumen Microbiome

The most recent authoritative text on the nutrition of major ruminants (NRC, 2016), states that the rumen is a “complex dynamic anaerobic ecosystem.” The dynamics of the microbial community arises from variability introduced by feed source, the environment, and physiological state impacts the microbiome (Xue *et al*., 2018). Experts (NRC, 2016) note that diurnal shifts of a full pH unit are not uncommon, and this can significantly impact the microbial population. The rumen microbial population is well adapted to these standard diurnal shifts in the rumen environment and continue to serve the function of digestion of feed despite these changes (NRC, 2016). This ability to rapidly adapt is due in part to the rumen microbiome’s ability to utilize specialized enzymes and enzyme complexes to convert feed components to end products of digestion and microbial cells (NRC, 2016). It is this specific understanding that ASCUS uses in their identification of existing, commensal microorganisms in the rumen of high producing ruminants. Particularly, understanding of their unique enzymatic properties and physiology support the selection and use of them as DFMs.

Several studies have linked the rumen microbiome profile to animal performance and digestibility (Lima *et al*., 2015; Jami *et al*., 2013; Kumar *et al*., 2015). The rumen microbiome is highly variable depending on several factors including age, breed, diet composition, time after feeding, season, stage of lactation, location, and farm management practices (Pitta *et al*., 2016; Furman *et al*., 2020; Henderson *et al*., 2015; Wallace *et al*., 2019). Additionally, there are groups of microorganisms that are unique to particular breeds of cow (i.e., Jersey or Holstein), regions, and individual animals that further increase the inherent complexity of the microbial community native to the rumen. Diet, in particular, has been shown to be the main driver of microbiome composition (Ghaffari *et al*., 2014). To better study the microbiome in context of this variability, many studies have focused on identifying and characterizing the core rumen microbiome (Petri *et al*., 2013; Xue *et al*., 2018; Henderson *et al*., 2015; Wallace *et al*., 2019; Furman *et al*., 2020; Kumar *et al*., 2015; Jami *et al*., 2013; Kittleman *et al*., 2013; Lima *et al*., 2015; Fouts *et al*., 2012). The concept of core microbiome, a common assemblage of microorganisms that exists in or is associated with a specific habitat, was first introduced and applied to differentiate human microbiomes associated with healthy and diseased conditions (Turnbaugh *et al*., 2007; Turnbaugh and Gordon, 2009; Turnbaugh *et al*., 2009). Since then, core microbiomes have been identified in a broad spectrum of environments including agroecosystems, monogastric animals, and ruminants (Shade *et al*., 2012; Yeoh *et al*., 2017; Toju *et al*., 2018; Lowe *et al*., 2012; Dougal *et al*., 2013).

There is a core microbiome that appears in the majority of dairy cows that provides the basal level of fermentation required for animal survival. Although the results are variable at times and defining a “normal healthy'' rumen is challenging, there are several phyla that tend to appear across all ruminants. Henderson *et al*. (2015) reported 32 different species of ruminants globally shared a core assembly of rumen bacteria. Xue *et al*. (2018) demonstrates that individual animals within a large cohort of dairy cattle with similar genetics, diet, environment, and management can have significant differences in their rumen microbiome species. The core microbiome identified included microorganisms from over 391 genera covering 26 phyla. The microorganisms unique to individual animals (termed “pan microbiome”) along with the core microbiome dictated the variability in rumen fermentation and production. Consistent with other studies (Jami, 2012; Lima, 2014; Deusch, 2017; Huws, 2018; Xue, 2018), members of Bacteroidetes, Firmicutes, Proteobacteria, and Fibrobacteres were among the topmost abundant bacteria identified regardless of animal origin and diet.

Experts (NRC, 2016) suggest that fungi, such as *Pichia* species, make up 8-20% of the microbial mass. Commensal rumen fungi are known to contribute significantly to fiber digestion in the rumen (NRC, 2016). The fungal rumen community, although much less abundant than the bacterial rumen community, tends to fall into the following phyla: Ascomycota, Basidiomycota, Neocallimastigomycota, and Zygomycota (Fouts, 2012; Dias, 2017; Paul, 2018; Belanche, 2019; Kittelmann, 2013; Lima, 2015; Mendes de Almeida, 2012; Vargas-Bello-Perez, 2016; Kumar, 2015; Ishaq, 2017; Tapio, 2017; Langda, 2019). Neocallimastigales used to be an order within Chytridiomycota, however in 2012, these anaerobic fungi were placed into a separate phylum called Neocallimastigomycota (Adl, 2012).

As more rumen microbiomes were studied, it became clear that diet was the major determinant of observed microbiome differences (Johnson, 1995; Brulc, 2007; Carberry, 2014; Deusch, 2017; Belanche, 2019; Kumar, 2015; Mizrahi, 2018). This indicates the direct impact of diet on rumen microbial populations. Hence, modifying either diet or microbiome could influence the rumen fermentation process (Morais, 2019; Wallace, 2019; Furman, 2020; Belanche, 2012). To better assess the potential impact for *P. kudriavzevii* ASCUSDY21 on deleteriously impacting the existing microbiome, 9 publicly available core rumen bacteria studies and 6 publicly available rumen core fungi studies were utilized to identify the upper and lower thresholds of key phyla in the core rumen microbiome of a healthy ruminant across a variety of conditions (Appendix 018). If the abundances of core microbiome members are within typically observed ranges, it is likely that rumen fermentation is also operating within normal ranges as well. These studies cover a variety of diets, as diet has the most impact on microbiome composition. In-house data corroborates that no large shifts in the core microbiome beyond observed thresholds are anticipated through feeding a native microorganism, and thus, no detrimental effects of rumen fermentation are expected (Appendix 018). The intent of feeding DFMs, particularly *P. kudriavzevii* ASCUSDY21, is to improve the nutrient availability from feed. Feeding *P. kudriavzevii* ASCUSDY21 to dairy cattle supplements the existing populations of *P. kudriavzevii* in the rumen, and ultimately provides additional enzymes that enable increased feed digestion and nutrient availability to the animal. Should *P. kudriavzevii* ASCUSDY21 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. This notice includes a more detailed discussion of the core microbiome and microbiome safety in Section 6.4 of this GRAS notice.

2.5.2 Impact of Failure of the Notified Substance

If this product fails, that is, the product fails to enhance rumen digestibility, there would not be a safety concern with respect to the animal's health or nutrition. The notified substance increases the digestion of insoluble starch and cellulose by acting upon the existing feed within the rumen. The diet offered to the animal would be formulated to meet the existing nutritional needs of the animal (NRC, 2001). Should *P. kudriavzevii* ASCUSDY21 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients.

The contribution of yeasts, specifically, to the fermentation characteristics of the rumen have been extensively evaluated in the published literature (Elghandour *et al*., 2015, Lynch and Martin 2002, Puniya *et al*., 2015, Chaucheyras-Durand, 2019). Use of *Saccharomyces cerevisiae* based products show a wide variability of responses on animal digestibility and performance. Several published meta-analyses have attempted to resolve these differences, as results from individual experiments tend to be very inconsistent. Ali-Haimoud-Lekhal *et al*. (1999) did not observe any significant differences on milk

production in late-lactation dairy cows being fed yeast-based supplements. Similarly, meta-analyses presented in Lescoat *et al*., 2000 and Sauvant *et al*., 2004 showed no effect of *S. cerevisiae* on rumen fermentation characteristics in cattle. This point has been exemplified in a number of published papers. Desnoyers, *et al*., (2009) provided a meta-analysis (covering 157 experiments) assessing the influence of *S. cerevisiae* supplementation of ruminal parameters, intake and production characteristics. Within this dataset, 116 experiments reported rumen characteristics data, and 141 experiments reported animal production data. The intent of this study was to only investigate the effect of live yeast products. However, nearly half of the studies utilized (91/157, 58%) in this meta-analysis fed AllTech's Yea-Sacc1026. Although Yea-Sacc1026 is presented as a source of viable *S. cerevisiae* culture, independent studies have shown that the product consists of ~7.7% viable cells (Duarte *et al.*, 2012). Thus, majority (92.3%) of the product being fed to animals in the studies utilized in this meta-analysis is dead / inactive. Also, 13 yeast culture products (dead yeast / fermentation by product) were mistakenly included in this meta-analysis. Desnoyers, *et al.* (2009) reports that although yeast supplementation increased milk production and improved rumen fermentation in several studies, results were often inconsistent. The meta-analysis presented confirms the large variability present between studies, however, because of the large numbers of manuscripts used in the analysis, they were able to extract some significant results. Overall, yeast supplementation was found to increase rumen pH, VFA concentration, and OM digestibility, and rumen lactate concentration tended to decrease. No impacts on health were noted, despite the low viability of *S. cerevisiae* being fed. Similarly, Poppy, *et al*. (2012) also provided a meta-analysis of the impact of feeding *S. cerevisiae* cultures on milk production and VFA assessment as the ultimate measurement of feed digestibility in 36 studies. Yeast cultures differ from live yeast products in that they are fermentation by-products, and do not rely on live yeast cells to induce a physiological effect. Like Desnoyers, *et. al* (2009), Poppy, *et al.* (2012) showed substantial variability in animal production benefits among the studies. Sub-group analysis revealed that peer-reviewed manuscripts showed less heterogeneity, but still did not exhibit statistically significant treatment effects. No health impacts were reported, despite feeding non-viable yeast. Collectively, these reviews show that many of the individual studies did not demonstrate a significant difference in milk production or rumen parameters despite feeding ineffective yeast daily for long periods of time. This suggests "failure" of the DFM to impact digestibility, but in no instance was a decrease in production, compromised health, or other safety concern noted.

A dairy cattle study describing the use of *S. cerevisiae* to induce feed intake (available energy) during the two-week period pre and post-partum period and to reduce metabolic diseases related to energy reduction (Robinson, 1997) did not demonstrate a response. However, even though the supplementation of the yeast "failed" and cows did not respond to the yeast supplementation as expected, the supplementation of *S. cerevisiae* did not impact the health parameters assessed: body weight, body condition score, milk production, or milk composition. A similar study was reported on mid-lactation cattle (Schingoethe *et.al*., 2004) in which dairy cattle averaging 105 days in milk were fed *S. cerevisiae*. The intent of the study was to assess the impact of supplementation of live yeast on dry matter intake and milk production. There was no increase in dry matter intake or milk production. The supplementation of *S. cerevisiae* did not impact the health parameters assessed: body weight, body condition score, milk production, and milk composition. Hence, these studies support the contention that failure of the yeast supplementation would not be linked to a safety concern as failure of the product created no observable difference between control and treated animals.

Several published experiments have directly investigated the impacts of DFMs by comparing groups of animals receiving a "dead" microbial against a variety of treatment conditions. Cunha, *et al*. (2019) compared heifers fed a basal diet against heifers fed the same basal diet containing a live yeast or inactive yeast supplement (2 different doses) in a 5x5 Latin square experimental design with 15-day periods. Live and dead yeasts were administered to the appropriate animals after each feeding through infusion directly into the rumen. No differences in digestibility were observed between the control, live yeast, or either of the inactive yeast doses. No differences were observed in feed intake nor animal behavior. Hence the inactive yeast did not alter the overall digestion of the feed, nor impact the health of the animals. Feeding inactive yeast did not decrease rumen function. Inactive yeast is an AAFCO authorized feed ingredient commonly used as a food additive in ruminant and non-ruminant feed.

Muscato, *et al*. (2002) evaluated the feeding of fresh and inactivated rumen fluid to calves in a series of four experiments. The animals were dosed daily with 8mLs of either fresh or inactivated rumen fluid obtained from a cannulated Holstein cow from 0-6 weeks of age. In the first experiment, calves were either fed a typical basal ration or the same basal ration supplemented with fresh rumen fluid. In the second experiment, calves were fed the basal ration with either the cell pellet of fresh rumen fluid, supernatant of fresh rumen fluid, or no addition. In the third experiment, calves were fed a basal ration or a basal ration supplemented with autoclaved rumen fluid. Autoclaving rumen fluid ensures microbial death, thus inactivating the biological component. The fourth experiment had a similar set-up to the third experiment, but rumen fluid was only fed for 5 days rather than 6 weeks. In the studies that evaluated autoclaved rumen fluid, the number of days of scouring were significantly decreased compared to the control. Similarly, the calves receiving autoclaved rumen fluid experienced higher gains in the first two weeks, but by the end of the experimental period there was no impact on growth. There were no differences in the outcomes of calves receiving fresh rumen fluid as compared to calves receiving autoclaved rumen fluid. This study suggests that the feeding of inactivated microorganisms does not decrease rumen function or create a safety concern when fed to animals.

The lack of safety impacts when the direct fed microbial is not restricted to yeasts. Similar results were observed in studies feeding *Lactobacillus acidophilus* (Raeth-Knight *et al*., 2007, Abu-Tarboush *et al*., 1996, Higginbotham *et al*., 1993, McGilliard and Stallings, 1997). In Weiss *et al*. (2008), dairy cows were supplemented with *Propionibacterium* P169 2 weeks before anticipated calving to 119 days in milk. Cows fed *Propionibacterium* P169 had lower concentrations of acetate and greater concentrations of propionate and butyrate compared to control cows. Treatment cows also produced similar amounts of milk with similar composition as cows fed the control diet and had similar body weights throughout the trial. Chiquette *et al*. (2008) fed *Prevotella bryantii* 25A to dairy cows in early lactation, and found that administration did not change milk yield, but tended to increase milk fat. This is in alignment with the increased acetate and butyrate concentrations observed in the rumen of treatment animals. In Chiquette *et al*. (2007), *Ruminococcus flavefaciens* NJ was fed to non-lactating dairy cows on either a high concentrate or a high forage diet daily. Cows fed *R. flavefaciens* NJ exhibited improved *in sacco* digestibility of hay in the rumen when fed as part of a high concentrate diet. Several experiments have fed *Megasphaera elsdenii* with various results on digestibility and performance, but no deleterious impacts were observed (Aikman *et al*., 2011, Hagg *et al*., 2010, Zebeli *et al*., 2012, Hagg *et al*., 2008, Kung, 1995)

In these examples, failure of DFM supplementation or the DFM itself did not cause any harm to the fermentation characteristics of the rumen or animal well-being. In the case of *P. kudriavzevii*

ASCUSDY21, if the DFM failed to provide improved digestibility, rumen fermentation of treated cows would be identical to rumen fermentation of untreated cows. Since no alterations are made to the standard feeding regime when using this product, the value of the feed that would be digested and utilized for the nutrients required to sustain life is identical between the control and treated group. Animals would be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001). Any non-performing *P. kudriavzevii* or deceased *P. kudriavzevii* would pass through the GI tract with the normal flow of digesta, providing nutrients for absorption by the animal (NRC, 2016). It is well understood that non-viable yeast (AAFCO 96.1, 96.3. 96.4, 96.5 and others) and non-viable microorganisms (AAFCO 36.6, 36.1, 36.7, 36.9 and others) are authorized as animal feed ingredients and are useful sources of nutrition in animal diets (AAFCO, 2020).

In this respect, based on the results of published comparative studies, *P. kudriavzevii* ASCUSDY21 will act only to support normal ruminal function of digestion of animal feed. Like other DFMs, while *P. kudriavzevii* ASCUSDY21 may aid the digestion of feed, the effect is not required for the general well-being and normal performance of dairy cattle. Thus, the absence of the anticipated effect of *P. kudriavzevii* ASCUSDY21 on feed digestion by dairy cattle would not have an impact on safety. ASCUS product labeling does not suggest a change in normal feeding regime, and its use would be specific for gaining additional nutritional value from a typical balanced ration. Animals would continue to be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001).

2.5.3 Summary

In summary it is ASCUS' understanding that the regulatory hurdle provided in §570.230(d), is not applicable to the conclusion of the generally recognized as safe substance *P. kudriavzevii* ASCUSDY21, that is "failure" of the intended use will not raise a safety concern, as the intended use is to provide increased nutritive value from nutritionally adequate feeds. Should *P. kudriavzevii* ASCUSDY21 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. Therefore, there is no regulatory requirement to provide specific utility data to support the intended use.

PART 3 – TARGET ANIMAL AND HUMAN EXPOSURE

3.1 Target Animal Exposure

3.1.1 Exposure to the Direct Fed Microbial Strain

As mentioned in Part 1, *P. kudriavzevii* ASCUSDY21 is intended for use as a source of viable microorganisms in feed for dairy cattle. The microbial strain will be delivered in the fat encapsulated form (i.e., fat encapsulated *P. kudriavzevii* ASCUSDY21, min. (b) (4)) to dairy cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing *P. kudriavzevii* ASCUSDY21 may be incorporated into the diet of dairy cattle include as part of the TMR, as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. The fat encapsulated product will be incorporated into dairy cattle feed at levels at a recommended intake level of $1x10^8$ CFU *P. kudriavzevii* ASCUSDY21/cow/day.

3.1.2 Exposure to the Other Components of the Fat Encapsulated Product

At the recommended intake level of $1x10^8$ CFU *P. kudriavzevii* ASCUSDY21/cow/day, the animal will be exposed to around 2.5 g of the fat encapsulated product (min. (b) (4)). As mentioned in Part 2.2.2, the amount of hydrogenated glycerides, sodium sulfate and freeze-dried *P. kudriavzevii* ASCUSDY21 in the fat encapsulated product are adjusted for each batch to standardize the viable cells count. These encapsulation ingredients are acceptable for use in dairy cattle feed and comply with the corresponding ingredient definitions in the AAFCO Official Publication (AAFCO, 2020; ingredient definitions 33.19 and 57.106 – see Appendix 011). Under these conditions of use, the animal will be exposed to in the region of 1.25 g of hydrogenated glycerides and 0.75 g of sodium sulfate. Considering that the typical dry matter (DM) intake by dairy cattle will be in the region of 25 kg/cow/day, the contribution of hydrogenated glycerides to the daily ration is expected to be no more than 0.005% DM. While the fat concentration of typical dairy diets is reported to be relatively low (approximately 2.5% DM), supplemental fats can be added to achieve a total ration fat content of around 6% DM (MSD Veterinary Manual, 2019). On this basis, the use of hydrogenated glycerides or similar acceptable fat source as an encapsulating aid in the manufacture of fat encapsulated *P. kudriavzevii* ASCUSDY21 will have a negligible impact on the total fat intake by dairy cattle under the conditions of use. Similarly, an intake of 0.75 g/cow/day of sodium sulfate will provide dairy cattle with approximately 0.24 g of sodium/cow/day, representing less than 0.001% of the DM intake. The maximum tolerable levels of sodium chloride set by the National Research Council (NRC) for lactating cows is 3% of DM intake, equivalent to around 1% DM of sodium. Thus, the use of sodium sulfate as an encapsulating agent in the manufacture of fat encapsulated *P. kudriavzevii* ASCUSDY21 is not expected to have any significant impact on the overall sodium intake by dairy cattle under the intended conditions of use. Another element of interest is sulfur. The use of *P. kudriavzevii* ASCUSDY21 would provide approximately 0.75 grams of sodium sulfate or 0.17 grams of sulfur per day. The NRC (2005) has suggested that Total Mixed rations (grain based) of cattle diets should be at a maximum tolerable level of 0.3% sulfur (75g/cow/day), as such this ingredient would provide an insignificant amount of the total sulfur in the diet of the dairy cow.

3.1.3 Background Exposure to the Microorganism

As mentioned in Part 2, the strain was isolated from the rumen content of a healthy mid-lactation Holstein cow and in this respect, *P. kudriavzevii* ASCUSDY21 will contribute to the native population of *Pichia* species in the gut of the animal (see Part 6). In this respect, *P. kudriavzevii* is one of the prevalent yeast species identified in the rumen of cattle and further details on the abundance of the species obtained from survey experiments are provided in Part 6 and Appendix 018.

Furthermore, *P. kudriavzevii* occurs widely in the environment and is commonly encountered in forages and silages used for feedstocks (Santos *et al*., 2015; Carvalho *et al*., 2017; Santos *et al*., 2017). While not present to a significant or intentional degree in these feedstocks, background exposure is likely to be continuous as part of the daily ration of dairy cattle.

3.2 Human Exposure

Fat encapsulated *P. kudriavzevii* ASCUSDY21 is intended for use as a supplemental source of viable microorganisms in the feed of dairy cattle. As mentioned in Part 2, the strain was isolated from the rumen content of a healthy mid-lactation Holstein cow and in this respect, *P. kudriavzevii* ASCUSDY21 will contribute to the native ruminal population of *Pichia* species (see Part 6). No transfer of viable *P. kudriavzevii* ASCUSDY21 from the rumen to milk or other edible tissues is anticipated.

Furthermore, *P. kudriavzevii* is ubiquitous in nature and has a long and established history of use in the production of traditional fermented foods for humans (e.g., as described by EFSA, 2007 and Douglass *et al*., 2018 – see Part 6). Thus, any exposure by humans unintentionally to viable *P. kudriavzevii* ASCUSDY21 from its use in feed is not expected to be significant compared to that from the environment and the consumption of fermented foods.

The strain has been unambiguously characterized as *P. kudriavzevii* and whole genome sequence analysis indicates the absence of any genetic element sequences that code for virulence factors or protein toxins (see Part 2, and Appendices 003 and 006). As a consequence, there should be no transfer of pathogenicity or toxigenicity to milk or edible tissues through the use of *P. kudriavzevii* ASCUSDY21 as a source of viable microorganisms in the feed of dairy cattle.

PART 4 – SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with *P. kudriavzevii* ASCUSDY21.

PART 5 – EVIDENCE BASED ON COMMON USE BEFORE 1958

Not applicable.

PART 6 – NARRATIVE

The conclusion that *P. kudriavzevii* ASCUSDY21, as described herein, is GRAS under the conditions of intended use as a direct fed microbial in feed for dairy cattle is based on scientific procedures using product-specific characterization data on the microbial strain together with a body of published information on the prevalence and potential pathogenicity of the *Pichia* species.

As mentioned in Part 1.3, *P. kudriavzevii* ASCUSDY21 will be provided to dairy cattle as a fat encapsulated product either alone or in combination with other direct fed microbials. The strain was isolated from the rumen content of a healthy mid-lactation Holstein cow and is intended as a source of live naturally occurring microorganisms. In this respect, *P. kudriavzevii* ASCUSDY21 will contribute to the digestibility of feedstuffs in the rumen. The functionality of the direct fed microbial strain is considered in Part 6.1.

The safety of *P. kudriavzevii* ASCUSDY21 for use as a direct fed microbial for dairy cattle is evaluated according to the guidelines developed by Pariza *et al*. (2015). These guidelines are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial cultures for consumption by humans and animals (AAFCO, 2020). In accordance with these guidelines, the safety of a microorganism without an extensive history of use in food or feed is primarily addressed by evaluating a series of questions. The microbial strain must be fully characterized and the body of knowledge pertaining to safety based on its taxonomic unit considered. Full details of the characterization of *P. kudriavzevii* ASCUSDY21 are detailed in Part 2. The microorganism has been unambiguously characterized as *Pichia kudriavzevii* (see Section 2.1.4 and Appendix 003). Furthermore, whole genome sequence analysis indicates the absence of any genetic element sequences that code for virulence factors or protein toxins (see Section 2.1.8 and Appendix 006), as well as transferable genetic elements (see Section 2.1.5 and Appendix 007). Whole genome sequence analysis together with phenotypic testing indicate that *P. kudriavzevii* ASCUSDY21 is susceptible to antifungals and should not increase the risk of transfer of resistance to other microorganisms (see Section 2.1.6, Appendix 004). Testing also confirms *P. kudriavzevii* ASCUSDY21 does not produce antimicrobial substances during fermentation (see Section 2.1.7 and Appendix 005). *P. kudriavzevii* ASCUSDY21 has not been genetically modified. Safety of this microorganism is based on the natural occurrence and prevalence of *P. kudriavzevii* as a commensal organism in the rumen of ruminants as well as in fermented foods, and characterization of the strain to indicate absence of any anticipated virulence factors for pathogenicity or antifungal resistance of concern.

In addition to the characterization data, a body of information is available in the public domain pertaining to (a) the identity and natural occurrence of *P. kudriavzevii* (see Part 6.2); (b) the history of exposure of the species by animals and humans (see Parts 6.4 and 6.5); and (c) the potential for toxigenicity and pathogenicity (see Part 6.6). Following the decision tree established by Pariza *et al*. (2015), these data are pivotal to the safety evaluation of *P. kudriavzevii* ASCUSDY21 and are summarized below. The Pariza *et al*. (2015) decision tree that outlines the safety evaluation is provided in Appendix 016.

6.1 Functionality

The microbial population of the rumen plays an important role in the utilization of feed by dairy cattle. Manipulation of rumen microbiota by dietary supplementation with sources of viable microorganisms is

common practice in the dairy cattle industry in the U.S. in order to facilitate fermentation and general digestive health of the animal (Yoon & Stern, 1995; Chaucheyras-Durand & Durand, 2010; El-Tawab *et al*., 2016). The contribution of yeasts generally to the fermentation characteristics of the rumen have been extensively evaluated in the published literature, with important functions reported to be stabilization of the rumen pH, stimulation and growth of cellulolytic bacteria, increase in volatile fatty acid production, reduction in ammonia concentrations, improved microbial protein synthesis and fiber digestibility (*e.g*., Erasmus *et al*., 1992; Newbold *et al*., 1998; Yoon & Stern, 1995; Bomba *et al*., 2002; Nocek *et al*., 2002; Denev *et al*., 2007; Bakr *et al*., 2015). As mentioned in Part 2, *P. kudriavzevii* ASCUSDY21 was isolated from the rumen content of a healthy mid-lactation Holstein and is expected to contribute in the same way as other yeasts to digestion and metabolism in the ruminal environment.

In particular, *P. kudriavzevii* ASCUSDY21 was shown to utilize various carbon sources including simple carbohydrates such as glucose, fructose, and glycerol, as well as more complex carbohydrates such as cellulose and starch (see Part 2.1). Similar phenotypes are reported in the published literature for other *P. kudriavzevii* strains (*e.g*., Yuangsaard *et al*., 2013; Arora *et al*., 2015). Thus, the microorganism has the potential to support digestion by aiding fermentation of forages and complex carbohydrates in the rumen.

It is widely recognized that feeding of highly concentrated diets to dairy cattle can result in an increase in lactic acid production and reduction in ruminal pH with a concomitant adverse effect on dairy cattle (Williams *et al*., 1991; AlZahal *et al*., 2008; Sirisan *et al*., 2013). *P. kudriavzevii* ASCUSDY21 along with other *P. kudriavzevii* strains have been demonstrated to utilize lactic acid as a source of carbon and energy and therefore, have the potential to help stabilize ruminal pH (Sirisan *et al*., 2013) and support normal digestion and metabolism.

Furthermore, *P. kudriavzevii* strains are widely reported to produce folate on fermentation of cereals, a functionality historically utilized to improve the nutritional value of cereal-based human foods (*e.g*., Korhola *et al*., 2014; Ogunremi *et al*., 2015; Greppi *et al*., 2017). Folate production has been demonstrated to be important in maintaining dairy cattle performance (Li *et al*., 2016; Graulet *et al*., 2007) and *P. kudriavzevii* ASCUSDY21 has the potential to play a role in maintaining ruminal concentrations of this nutrient through the metabolism of dietary cereals.

A number of *P. kudriavzevii* strains also have been demonstrated to secrete phytase (Hellström *et al*., 2012; Nuobariene *et al*., 2011; Chan *et al*., 2012; Greppi *et al*., 2015; Hellström *et al*., 2015). Phytate is degraded by phytase enzymes largely of microbial origin in the rumen of cattle to release phosphorus for absorption and may contribute to this enzymatic activity.

P. kudriavzevii, along with a number of other fungi, have also been investigated and fed to animals for their ability to bind aflatoxins (Intanoo *et al*., 2020; Intanoo *et al.*, 2018). Intanoo *et al.*, 2018 isolated a number of fungi from the rumen fluid of fungi. Isolates were screened for their ability to detoxify aflatoxin B_1*.* *P. kudriavzevii* was found to detoxify up to 85% of aflatoxin B_1 *in vitro.*

Taken together, these examples of the potential functionality of *P. kudriavzevii* in the rumen support the proposed role of *P. kudriavzevii* ASCUSDY21 as a source of viable microorganisms in the diet to help support digestion and metabolism in the rumen. While *P. kudriavzevii* ASCUSDY21 may contribute to the native population of *Pichia* species in the gut of the animal, the technical function has no bearing on the safety when used as a direct fed microbial in feed for dairy cattle. On this basis, no further

demonstration of the technical effect (utility) of *P. kudriavzevii* ASCUSDY21 was required for the safety evaluation (see Part 2.5).

6.2 Identity

Population genomics was recently used by Douglass *et al*. (2018) to identify *P. kudriavzevii*, *Candida krusei*, *Issatchenkia orientalis* and *Candida glycerogenes* as the same species within the *Pichia* genus. Historically, the genus *Pichia* was one of the largest yeast genera, comprising nearly 100 species defined by phenotype. However, developments in gene sequencing has reduced the number of species to around 20 following reassembly of the phylogenetic tree (Kurtzmann, 2011; Brandt & Lockhart, 2012). In addition to these four commonly used names, a fifth synonym appears to be used in the published literature, *Candida acidothermophilus* (*e.g*., Subramanya *et al*., 2017). All potential nomenclature was included in literature searches to identify the body of available information pertinent to the safety of *P. kudriavzevii*.

6.3 Literature Search

A comprehensive literature search was conducted in order to identify all publicly available information pertaining to the safety of *P. kudriavzevii* for the intended use as a source of viable cells for dairy cows. As mentioned above in Part 6.2, all potential nomenclature were included in the literature search (*i.e*., *P. kudriavzevii*, *Candida krusei*, *Issatchenkia orientalis, Candida acidothermophilus* and *Candida glycerogenes*) and details of the search strategy are provided in Appendix 017.

A systematic search was conducted using Web of Science up to November 6, 2019. From the pertinent studies identified, searches were conducted for citations not captured in the initial search. Scientific opinions by authoritative bodies as well as reviews were also evaluated as a final check for the completeness of the search. The data summarized below are considered representative of the available body of information.

6.4 Natural Occurrence

6.4.1 Prevalence in Animals

P. kudriavzevii is one of the prevalent yeasts identified in the rumen of cattle (Lund, 1974; Lund, 1980; de Almeida *et al*., 2012; Sirisan *et al*., 2013; Intanoo *et al.*, 2018; Fernandes *et al*., 2019) and the gastrointestinal tract of poultry (García-Hernández *et al*., 2012; Magnoli *et al*., 2016; Subramanya *et al.,* 2017). Analyses of the rumen fluid from Holstein cows and heifers fed different forages in Brazil identified 38 yeast isolates of which 32 isolates corresponded to the species *P. kudriavzevii* (de Almeida *et al*., 2012). Yeast populations were reported to be higher in animals fed chopped sugar cane than in those fed sorghum silages, postulated by the authors to relate to the type of carbohydrates available; yeasts are better able to degrade the simple carbohydrates found in forages such as sugar cane. In another study by Sirisan *et al*. (2013), *P. kudriavzevii* was one of the three most effective lactic-acid utilizing yeasts in terms of specific growth rate in dairy cattle fed high cassava pulp diets. Intanoo *et al.* (2018) isolated several yeast strains from the rumen fluid of dairy cattle to search for potential probiotic strains that were capable of detoxifying aflatoxin B_1. *P. kudriavzevii* was one of the species isolated directly from rumen fluid. Fernandes *et al*., 2019 also took a similar approach and isolated several microbial species directly from rumen fluid to identify potential probiotics. *P. kudriavzevii* was one of the

primary yeast species isolated in these experiments. Overall, *P. kudriavzevii* is naturally abundant in the gastrointestinal tract of healthy animals and not associated with any health concerns.

6.4.2 Microbiome Safety

The use of *P. kudriavzevii* to facilitate the digestion of complex carbohydrates such as starch and cellulose of animal feed within the rumen, as the enzymes of interest are related to amylase and carboxymethylcellulose. *P. kudriavzevii* has been fed to dairy cattle previously. Intanoo, *et al*. (2020) fed *P. kudriavzevii* and *Kluyveromyces marxianus* to dairy cows and found that supplementation improved detoxification of aflatoxin B_1 and improved DMI and milk components. No impacts on animal health were observed. The contribution of DFMs to the fermentation characteristics of the rumen has been extensively evaluated (Elghandour, 2015).

Microorganisms that are commonly used in DFMs for ruminants are lactic acid producing bacteria or lactic acid utilizing bacteria (Elghandour, 2015). Specific species within the genera *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus, Bacillus, Propionibacterium, Megasphaera and Prevotella* have also been fed to animals (Nocek *et al*., 2002; Yoon and Stern, 1995; Ghorbani *et al*., 2002; Stein *et al*., 2006; Yang *et al*., 2004; Nagaraja *et al*., 1997; Chiquette *et al*., 2008; Mohammed *et al*., 2012; Weiss *et al*., 2008; Aikman *et al*., 2011). There are several studies, for example, that describe the fermentation patterns and feed digestibility of ruminants fed a standard diet supplemented with a DFM compared to ruminants only on a standard diet. Feeding of *Lactobacillus plantarum* via silage in Mohammed *et al*., (2012) showed no changes in production, but no deleterious effects on the animal. Similar results were observed in studies feeding *Lactobacillus acidophilus* (Raeth-Knight *et al*., 2007, Abu-Tarboush *et al*. 1996, Higginbotham *et al*. 1993, McGilliard and Stallings, 1997). In Weiss *et al*. (2008), dairy cows were supplemented with *Propionibacterium* P169 2 weeks before anticipated calving to 119 days in milk. Cows fed *Propionibacterium* P169 had lower concentrations of acetate and greater concentrations of propionate and butyrate compared to control cows. Treatment cows also produced similar amounts of milk with similar composition as cows fed the control diet and had similar body weights throughout the trial. Chiquette *et al*. (2008) fed *Prevotella bryantii* 25A to dairy cows in early lactation, and found that administration did not change milk yield, but tended to increase milk fat. This is in alignment with the increased acetate and butyrate concentrations observed in the rumen of treatment animals. In Chiquette *et al*. (2007), *Ruminococcus flavefaciens* NJ was fed to non-lactating dairy cows on either a high concentrate or a high forage diet daily. Cows fed *R. flavefaciens* NJ exhibited improved *in sacco* digestibility of hay in the rumen when fed as part of a high concentrate diet. Several experiments have fed *Megasphaera elsdenii* with various results on digestibility and performance, but no deleterious impacts were observed (Aikman *et al*., 2011, Hagg *et al*., 2010, Zebeli *et al*., 2012, Hagg *et al*., 2008, Kung, 1995).

The contribution of yeasts, specifically, to the fermentation characteristics of the rumen have been extensively evaluated in the published literature (Elghandour *et al*., 2015; Lynch and Martin, 2002; Puniya *et al*., 2015; Chaucheyras-Durand, 2019). Use of *Saccharomyces cerevisiae* based products show a wide variability of responses on animal digestibility and performance. Several published meta-analyses have attempted to resolve these differences, as results from individual experiments tend to be very inconsistent. Ali-Haimoud-Lekhal *et al*. (1999) did not observe any significant differences on milk production in late-lactation dairy cows being fed yeast-based supplements. Similarly, meta-analyses presented in Lescoat *et al*. (2000) and Sauvant *et al*. (2004) showed no effect of *S. cerevisiae* on rumen

fermentation characteristics in cattle. The meta-analysis presented in Poppy *et al*. (2012) analyzed thirty-six studies feeding yeast culture based upon *S. cerevisiae.* This analysis suggests milk yield and energy-corrected milk yield increases of an average of 1.1 and 1.6 kg/d, respectively. Another meta-analysis conducted involving 110 papers covering 157 experiments feeding either yeast culture or live yeast products based upon *S. cerevisiae* (Desnoyers *et al*., 2009) suggested milk yield increases of about 0.6 kg/d (~ 1.1 g/d per kg of BW). Yeast supplementation also increased rumen pH, rumen VFA concentration, and organic matter digestibility was also increased by yeast supplementation. Although results are inconsistent, no health impacts were observed in any meta-analysis.

To corroborate the published literature (6.4.1) ASCUS Biosciences conducted a series of experiments in order to obtain a representative sampling of the rumen microbial community in dairy cows under farm-like conditions in the U.S. The full study report is provided in Appendix 018.

In two general survey experiments, animals were cannulated and sampling conducted across the different regions of the rumen over a number of days. In a third study, *P. kudriavzevii* ASCUSDY21 along with another native rumen microorganism was administered to lactating dairy cows via injection and rumen sampling conducted over a number of days. In the experiments performed by ASCUS Biosciences, the typical abundance of *P. kudriavzevii* specifically, in the rumen of dairy cows was found to vary from approximately 0.0001% to 20% of the fungal population. General observations indicated that all animals were in good health. *P. kudriavzevii* ASCUSDY21 inoculation was not observed to have a significant impact on the ruminal microbial community. Taken together, these studies provide corroborative experimental evidence that *P. kudriavzevii* is naturally abundant in the rumen of dairy cattle and not associated with any health concerns.

6.4.3 Environmental Occurrence

P. kudriavzevii occurs widely in the environment and is commonly encountered in soils, plants, and water sources. As stated in the "Census of Yeasts Isolated from Natural Ecosystem and Conserved in Worldwide Collections", nearly 3,000 members of *Pichia*, including *P. kudriavzevii*, have been deposited in culture collections around the world. These samples have been isolated from a diverse range of environments from all continents including plants, insects/invertebrates, aquatic habitats, mountains, fresh water, sea water, and soil. Extreme warm/dry and extreme cold habitats (glaciers, rocks, mountains) were also included in the list (Groenewald *et al*., 2017). Del Monaco *et al.* (2016) identified *P. kudriavzevii* as a regional member of the winemaking terroir of North Patagonia by sequencing environmental samples and comparing them to yeasts identified in wine. Abigail *et al.* (2013) isolated a *P. kudriavzevii* capable of degrading atrazine from an agriculture soil sample. Dhaliwal *et al.* (2011) isolated a thermotolerant *P. kudriavzevii* from sugarcane juice. *P. kudriavzevii* has also been identified in peat swamp forests, (Boonmak *et al.,* 2019) as well as other aquatic habitats (Hagler *et al.* 2017). The presence of *P. kudriavzevii* is widespread and not generally associated with any pathogenicity concerns.

6.4.4 Section Summary

P. kudriavzevii occurs widely in the environment and is prevalent in the rumen microbiome. Supplementation of the diet with *P. kudriavzevii* ASCUSDY21 will not negatively impact the function of the rumen nor negatively impact the well-being of the animal.

6.5 History of Use in Manufacture of Food and Feed Ingredients

Pichia species, including *P. kudriavzevii* are ubiquitous and have a long and established history of use in the production of traditional fermented foods (EFSA, 2007; Douglass *et al*., 2018). Examples of traditional foods in which *P. kudriavzevii* has been identified as a common or dominant yeast associated with the fermentation process include sourdough (Huys *et al*., 2013 cited in: De Vuyst *et al*., 2016), ghee (Ongol & Asano, 2009), the Brazilian non-alcoholic fermented cassava beverages tarubà (Ramos *et al*., 2015), yakupa (Freire *et al*., 2014), the West African fermented milk beverage nunu (Akabanda *et al*., 2013), cassava food lafun (Padonou *et al.,* 2009), the Chinese fermented cereal gruel suanzhou (Qin *et al*., 2016), Kazak artisanal cheese (Zheng *et al*., 2018), as well as various Asian and African alcoholic beverages (Li *et al.*, 2013, Thanh *et al*., 2016, Bi *et al*., 2016). Kuncharoen *et al.* (2020) isolated a number of yeasts from a variety of food and waste sources. They identified *P. kudriavzevii* in palm cakes, fermented beef, fermented fish, and fermented pork sausage.

The Joint Action Team of the Standing Committee on Microbiological Hygiene (SCMH) and the Standing Committee on Nutrition and Health (SCNH) published a scientific rationale for the inventory of microbial food cultures demonstrated as safe for use in food product(s) (Bulletin of the IDF No. 495/2018). In 2017/2018, this committee reviewed the 2012 published rationale and available taxonomic developments to update the inventory of microbial food cultures. Based on this guidance, *P. kudriavzevii* is also approved for use in dairy, wine, and coca products (Padonou *et al*., 2010; Daniel *et al*., 2009; Bai *et al*., 2010; Li *et al*., 2010; El-Sharoud *et al*., 2009; Osorio-Cadavid *et al*., 2008; del Monaco *et al*., 2014). *C. krusei* has been approved for use in wine (Bulletin of the IDF No. 495/2018; Charoenchai *et al*., 1997).

Furthermore, *P. kudriavzevii* is commonly encountered in forages and silages used for livestock feeding (Santos *et al*., 2015; Carvalho *et al*., 2017; Santos *et al*., 2017). Wang *et al*. (2018), for example, isolated strains of *P. kudriavzevii* from both whole crop corn silages and TMR silages. While the species is not substantial or characterizing in these livestock feeds as defined by Pariza *et al*. (2015)[1], their presence is widespread and not associated with any pathogenicity concerns.

A number of *Pichia* species have a history of use by the biotechnology industry for use in the manufacture of ingredients for use in food and feed (EFSA, 2007). In the EU, *P. angusta*, *P. anomala* and *P. jadinii* have been assigned qualified presumption of safety (QPS) status for the production of enzymes for use in food and feed (EFSA, 2007; 2008; 2009 and 2010). There is history of use, and a growing interest in the use of *P. kudriavzevii* in the manufacture of glycerol and other chemicals (Wang *et al*., 2001; Xiao *et al*., 2014; Radecka *et al*., 2015).

6.6 Potential for Toxigenicity and Pathogenicity

The potential pathogenicity of yeasts, including *P. kudriavzevii,* is widely reported in the published literature primarily under the name *C. krusei* (EFSA, 2008 and 2010). Similar to other yeasts, *P. kudriavzevii* is considered an opportunistic pathogen in humans and animals. The American Type Culture Collection (ATCC) lists *P. kudriavzevii* as BSL-1, indicating that it is a low-risk microorganism that poses little to no threat of infection in healthy humans and animals. DSMZ also classifies *P. kudriavzevii* as BSL-1. The Dutch Bureau for GMOs (COGEM) groups microorganisms into 4 pathogenicity classes,

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[1] Substantial and characterizing refers to species which are present to a significant and intentional degree, and have a measurable impact on the food properties (*e.g*., texture, flavor) (Pariza *et al*., 2015).

ranging from Class 1 to Class 4.The Dutch Bureau for GMOs (COGEM) lists *P. kudriavzevii* as Category 2A, indicating that it can cause disease in animals, but it is unlikely to spread within the population while an appropriate prophylaxis, treatment, or control strategy exists (COGEM, 2018). Similarly, according to the established rules for classifying biological agents, the Scientific Institute of Public Health in Belgium has assigned *C. krusei* to Category 2 as an agent presenting at the wild state a biological risk for immunocompetent humans and/or animals (Scientific Institute of Public Health, 2008).

There are a number of reports of *P. kudriavzevii*/*C. krusei* being associated with fungaemia and related infections in organs and oral cavities in humans (*e.g*., Merz *et al*., 1986; Nguyen *et al*., 1996; Vincent *et al*., 1998; Abbas *et al*., 2000; Singh, 2001; Krcmery & Barnes, 2002; Hachem *et al*., 2008; Al-Rawahi & Roscoe, 2013; Papon *et al*., 2013; Aslani *et al*., 2018; Bukamur *et al*., 2018). These infections are generally in individuals of weak health, particularly immunocompromised individuals with underlying serious illness. Factors demonstrated to increase the likelihood of infection are surgery, especially of the gastrointestinal tract, the fitting of catheters and treatment with immunosuppressive or chemotherapy drugs (Hazen, 1995; Abbas *et al*., 2000; Krcmery & Barnes, 2002; Tortorano *et al*., 2006; EFSA, 2008 and 2010; Al-Rawahi & Roscoe, 2013). In 2014, there was an outbreak of nosocomial sepsis in a neonatal intensive care unit due to *P. kudriavzevii* (Nagarathnamma *et al.*, 2017). Nine cases of sepsis were documented. Of these 9 cases, 7 were treated with voriconazole, and the patients were later discharged after improvement in condition. The other two patients went against medical advice and could not be followed up. Although *Candida* is typically the causative agent of sepsis, infections caused by *P. kudriavzevii* are more rare. Nagarathnamma *et al.* (2017) is considered the first report of neonatal sepsis due to *P. kudriavzevii.* Infections are normally treated with anti-fungal agents, although the resistance to antimycotics is an ongoing issue for *C. krusei* and other *Candida* species (Abbas *et al*., 2000; Krcmery & Barnes, 2002; Tortorano *et al*., 2006; Pfaller *et al*., 2008; Sardi *et al*., 2013).

The mechanisms by which fungi exhibit pathogenesis are poorly understood (EFSA, 2008). A number of reviews have considered the biology, epidemiology, pathogenicity and clinical manifestations of opportunistic *Candida* spp. (Samaranayake & Samaranayake, 1994; Mohandas & Ballal, 2011; Papon *et al*., 2013; Sardi *et al*., 2013). The available data indicate that the structural and metabolic characteristics of *C. krusei* are significantly different to that of other *Candida* species. *C. krusei* appears to be less virulent than *C. albicans* in terms of its ability to adhere to epithelial and prosthetic surfaces, and its ability to produce and secrete degradative enzymes (proteinase and phospholipase). In dairy cattle, bovine mastitis is normally caused by bacterial species but yeasts are reported to account for up to around 12% of cases (Krukowski *et al*., 2001; Wawron *et al*., 2010). *Candida* species, including *C. krusei* are commonly associated with incidences of mycotic mastitis, and considered to be opportunistic, occurring primarily in immunosuppressed animals (Krukowski *et al*., 2001; Wawron *et al*., 2010; Hayashi *et al*., 2013).

C. krusei / *P. kudriavzevii* is a potential opportunistic pathogen. It is estimated to be responsible for about 2% of yeast infections caused by *Candida* species in humans (Douglass *et al.,* 2018). There are roughly 700,000 cases of invasive candidiasis per year (Bongomin *et al.,* 2017), thus there are roughly 14,000 cases of *P. kudriavzevii*-based candidiasis per year. Assuming 7.8 billion people in the world as of May 2020, this rate of infection would imply that *P. kudriavzevii* may impact 0.00018% of the total population worldwide annually. Despite the wide prevalence and exposure of *P. kudriavzevii* in the environment and food, the number of annual infections is low. There are reports of yeasts exhibiting antifungal resistance, and phenotypic testing of *P. kudriavzevii* ASCUSDY21 has been conducted by

ASCUS and an independent testing facility to confirm the susceptibility of the strain to antimycotics of veterinary and pharmaceutical relevance (see Part 2.1 and Appendix 004). *P. kudriavzevii* ASCUSDY21 is not resistant to clinically relevant antimicrobial or antimycotic compounds. Thus, should *P. kudriavzevii* ASCUSDY21 cause an opportunistic infection in a human or animal, it can be readily treated using standard antibiotics.

6.6.1 Summary

Overall, the available information indicates that *P. kudriavzevii* / *C. krusei* is an opportunistic pathogen associated with infections in immunocompromised humans and animals. Despite the wide prevalence and exposure to *P. kudriavzevii* in the environment and food, the estimated number of cases of invasive candidiasis caused by *P. kudriavzevii* is low (~14,000 cases annually worldwide). As indicated in Part 2.1, interrogation of the whole genome sequence of *P. kudriavzevii* ASCUSDY21 did not reveal the presence of any genetic element sequences that code for virulence factors or protein toxins (see Appendix 006).

6.7 Studies in Target Animals

The determination that *P. kudriavzevii* ASCUSDY21 is GRAS under the intended conditions is based on product-specific characterization data together with the body of information in the published literature. The organism is a commensal rumen organism. One published study conducted by an independent researcher has directly administered *P. kudriavzevii* to dairy cattle. Intanoo, *et al*. (2020) fed *P. kudriavzevii* and *Kluyveromyces marxianus* to dairy cows and found that supplementation improved detoxification of aflatoxin B_1 and improved DMI and milk components. No impacts on animal health were observed.

Two investigative studies in which dairy cattle were inoculated with *P. kudriavzevii* ASCUSDY21 were conducted by ASCUS Biosciences corroborate the target animal safety determination. These unpublished studies, summarized in turn below, were of short duration and were designed primarily to assess the potential value of *P. kudriavzevii* ASCUSDY21 and other microorganisms as potential direct fed microbials. In both studies, *P. kudriavzevii* ASCUSDY21 was administered via ruminal cannulation in combination with at least one other microorganism. Overall, the study findings provide corroborative evidence that *P. kudriavzevii* ASCUSDY21 is well-tolerated and without adverse effects but are of limited relevance to the assessment of safety.

6.7.1 Study DUS1601 (Unpublished Study Report – Appendix 019)

In the first study, 16 multiparous Holstein cows were individually housed for a total of 52 days at (b) (4)). Animals underwent ruminal cannulation surgery followed by a 10-day recovery and adaptation period. After this time, the cows were allocated at random to one of 2 treatment groups (8 cows/treatment; 1 cow/replicate) and administered either buffer (control) or buffer containing a selection of microorganisms including *P. kudriavzevii* ASCUSDY21 once daily via ruminal cannulation for 32 days. Cows were monitored for a further 10 days after the last inoculation day. Observations included feed intake, body weight, milk yield, rumen digesta microbial content and pH, and fecal analysis. No adverse effects were reported for any of the variables measured over the duration of the study. Overall, the findings of the study corroborate the safety of *P. kudriavzevii* ASCUSDY21 for dairy cattle.

6.7.2 Study DUS1701 (Unpublished Study Report – Appendix 020)

In the second study, 32 Holstein cows approximately 100 days in milk, were assigned to one of 3 treatment groups (8 cows/treatment; 1 cow/replicate; mean days in milk, approximately 100). Cows were administered a buffer containing either 2 (treatment group 1), 3 (treatment group 2) or no (treatment group 3; control) microorganisms once daily via direct injection into the rumen for 28 days. The microorganisms fed to the cows in treatment groups 1 and 2 included *P. kudriavzevii* ASCUSDY21. Fecal samples were taken at Days 1, 8, 16, 24 and 28 and analyzed for neutral detergent fiber (NDF), acid detergent fiber (ADF) and DM content. Rumen contents also were sampled from each cow at Days 1, 8, 18, 24 and 28. From Day -7 to Day 38 of the study, observations included milk yield, general health and clinical udder evaluation.

Abnormal clinical udder findings and abnormal clinical health observations were considered minimal, incidental and not related to treatment for the duration of the study. No adverse effects were reported in any of the other variables measured for the duration of the study. Overall, the findings of the study corroborate the safety of *P. kudriavzevii* ASCUSDY21 for dairy cattle.

6.8 Summary and Critical Evaluation of Target Animal Safety

P. kudriavzevii is one of the prevalent yeast species identified in the rumen of cattle and gastrointestinal tract of poultry and is present naturally in forages and silages used in livestock feeding. *P. kudriavzevii* is widely prevalent in the environment and food. There is a long and established history of safe use of *P. kudriavzevii* strains in the production of traditional fermented foods, such as sourdough, fermented milks, and fermented cereal products around the world. Similar to other yeasts, *P. kudriavzevii* (reported as *C. krusei*) is an opportunistic pathogen with the potential to cause infection in immunocompromised individuals. This organism has been previously fed to dairy cattle with no deleterious health effects (Intanoo, *et al.* 2020 and Appendix 18). ASCUS Biosciences has interrogated the whole genome sequence of *P. kudriavzevii* ASCUSDY21 and confirmed the absence of any genetic elements that code for virulence factors (see Part 2.1 and Appendix 006). Moreover, the susceptibility of *P. kudriavzevii* ASCUSDY21 to antifungals of veterinary and pharmaceutical relevance has been demonstrated (see Part 2.1.6 and Appendix 004). There are no hazards identified specific to impurities or contaminants of the intended product (see Part 3.1.2). Collectively, these data indicate that *P. kudriavzevii* ASCUSDY21 should not be associated with any safety concerns for dairy cattle under the intended conditions of use as a direct fed microbial. Use of the Pariza (2015) decision tree for ensuring the safety of the consumption of microorganisms in human and animal diets supports the safety assessment. Safety of this microorganism is based on the natural occurrence and prevalence of *P. kudriavzevii* as a commensal organism in the rumen of ruminants as well as in fermented foods, and characterization of the strain to indicate absence of any anticipated virulence factors for pathogenicity or antifungal resistance of concern

In this safety assessment we identified, discussed and placed into context data and information that are, or may appear to be inconsistent with the GRAS status (21 CFR 570.250(c)(1)).

6.9 Summary and Critical Evaluation of Human Food Safety

Fat encapsulated *P. kudriavzevii* ASCUSDY21 is intended for use as a supplemental source of viable microorganisms in the feed of dairy cattle. While the microbial strain will contribute to the existing

fungal population in the rumen, there should be no transfer from the rumen to edible tissues, including milk. Furthermore, the strain has been unambiguously characterized as *P. kudriavzevii* and whole genome sequence analysis indicates the absence of any genetic element sequences that code for virulence factors or protein toxins (see Part 2.1). The absence of pathogenicity or toxigenicity is supported by the ubiquitous nature of *P. kudriavzevii* and the long and established safe history of use in species for the production of traditional fermented foods for humans. Taken together, these data indicate that *P. kudriavzevii* ASCUSDY21 should not be associated with any human food safety concerns under the intended conditions of use as a direct fed microbial in the feed of dairy cattle.

In this safety assessment we identified, discussed and placed into context data and information that are, or may appear to be inconsistent with the GRAS status (21 CFR 570.250(c)(1)).

PART 7 – LIST OF SUPPORTING DATA AND INFORMATION IN YOUR GRAS NOTICE

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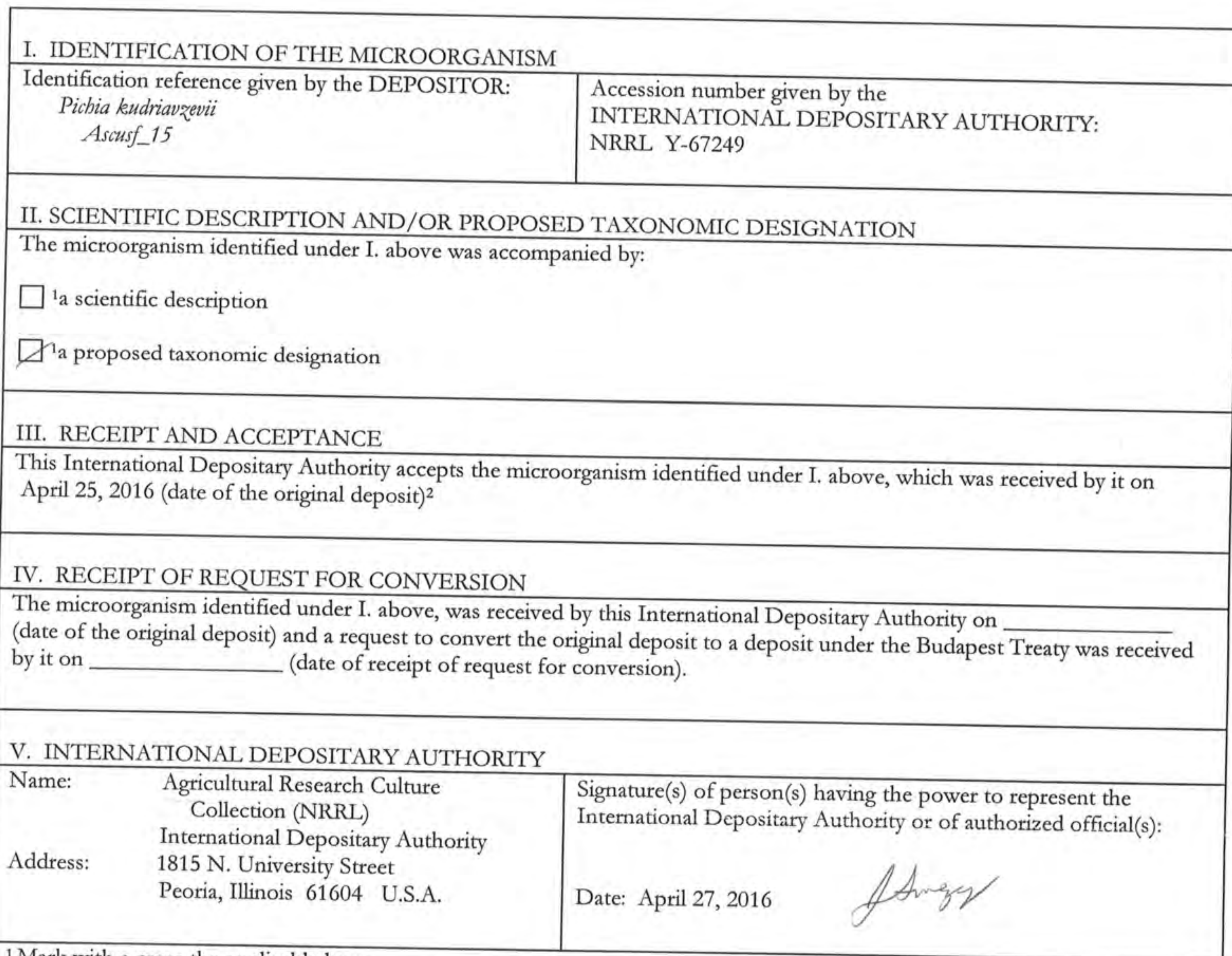
BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSE OF PATENT PROCEDURES

INTERNATIONAL FORM

TO
Patent Dept.
Ascus Biosciences
6450 Lusk Blvd. E102/209
San Diego, CA 92121

NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page



I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Pichia kudriavzevii *Ascusf_15*	NRRL Y-67249

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

☐ [1]a scientific description

☒ [1]a proposed taxonomic designation

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on April 25, 2016 (date of the original deposit)[2]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I. above, was received by this International Depositary Authority on ______________ (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on ________________ (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Agricultural Research Culture Collection (NRRL) International Depositary Authority Address: 1815 N. University Street Peoria, Illinois 61604 U.S.A.	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: April 27, 2016

[1] Mark with a cross the applicable box.

[2] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Objectives

The objective of this work was to assess the carbohydrate degradative capabilities of *Pichia kudriavzevii* ASCUSDY21 through genomic sequencing and *in vitro* digestion assays.

Methods

To determine carbon source utilization of Arabinose, Ribose, Mannitol, Sorbitol, Gluconate, Xylose, Cellobiose, and Starch, (b) (4) (b) (4) (b) (4) All of the carbon sources were filtered sterilized prior to adding to post-autoclaved media, except for cellobiose and starch, which were added prior to autoclaving. Prior to autoclaving the media was pH adjusted to (b) (4) of a single *P. kudriavzevii* colony that was inoculated into 10 mL of RAMM salts buffer was added to their respective well. *P. kudriavzevii* was incubated at 37C. OD600 readings were taken prior to incubation. *P. kudriavzevii* was incubated for 166.5 hours. After incubation OD600 was measure again to determine organism growth.

To determine carbon source utilization of Fructose, Dextrose, Lactose, Maltose, Sucrose, Cane Molasses, Beet Molasses, Lactate, Succinate, and Glycerol, the following growth media was prepared per 500 mL: (b) (4) (b) (4) The pH of the media was then adjusted to (b) (4) and sparged for 1 hour with (b) (4) after which the media was then autoclaved at 121C for 20 minutes. After autoclaving the media was moved to an anaerobic environment and (b) (4) (b) (4) Each of the following carbon sources were prepared at 20 g per 100 mL, sparged with (b) (4) gas and autoclaved: dextrose, lactose, maltose, sucrose, cane molasses, beet molasses, fructose, glycerol. Acetate was added at 50 mM and sparged with (b) (4) gas for 20 minutes and autoclaved. Lactic Acid and succinic acid were at a concentration of 10g/100 mL, pH adjusted to (b) (4) for 20 minutes then autoclaved. 0.8 mL of the above media was added to each well of an anaerobic 96 well plate. 0.08 mL of each carbon source was added to their respective well. 8 µL of preculture was then added to the necessary wells. The samples were then incubated at 37C for 48 hours. At 24 hours and 48 hours post inoculation 80 µL of culture was removed and the OD600 was measured.

To determine carbon source utilization of complex carbohydrates, 0.1 g of the following carbon sources (cellulose, cellulose paper, glucose, grass, bark, reed canary grass, starch, corn stover, (b) (4) (b) (4) (b) (4) The pH was then adjusted to (b) (4) and autoclaved at 121C for 20 minutes. (b) (4) (b) (4) *P. kudriavzevii* inoculum was then added to each tube at 1% v/v and incubated at 37C for 8 days on a shake plate. After incubation 0.5mL of culture were added to a 96 well plate and centrifuged at 750 rpm for 5 minutes. After centrifugation, 100 µL of supernatant was removed and aliquoted into 96 well plate where the OD 600 was then measured.

To determine carbohydrate active enzymes (CAZymes) present in the genome of *P. kudriavzevii*, genomic DNA was isolated via bead-based lysis using the MoBio PowerViral DNA kit (Carlsbad, CA). Sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA), and the resulting libraries were

paired-end sequenced (2x300bp) on an Illumina Miseq. The draft genome was assembled using SPAdes [version 3.6.2] (Bankevich et al., 2012). The open reading frames were predicted through AUGUSTUS using all deposited mRNA sequences for *P. kudriavzevii* in NCBI for training (Stanke and Morgenstern 2005). Predicted genes were annotated through the Pfam (Finn et al., 2016) database and UniRef50 Database (Suzek et al., 2014) using DIAMOND (Buchfink et al., 2015). Carbohydrate active enzymes were identified by Pfam domain description.

To determine carbohydrate-degrading activity, a colorimetric DNS assay (Xiao et al., 2005) was used. Triplicate cultures were inoculated from the *P. kudriavzevii* working cell bank (DY21 WCB-1 12DEB18 MS) and were grown in (b) (4) soluble starch, (b) (4) yeast extract, and (b) (4) peptone for 24.5 hours EFT (late exponential growth phase). Supernatants were assessed for activity against the indicated substrate in a reaction mixture at pH (b) (4) and 37ºC by subtracting the signal at time 0 from the signal at one hour of reaction time. Reaction substrates included soluble starch (Sigma Aldrich) or sodium carboxymethylcellulose (Sigma Aldrich) at (b) (4) (w/v) concentration in a (b) (4) phosphate/citrate buffer at pH (b) (4) Activities are reported as the average of the values from the triplicate cultures.

Results

P. kudriavzevii was assessed for growth on a variety of carbon sources. The carbon source utilization data is shown in Table 1.

Table 1: Growth of *Pichia kudriavzevii* on Different Carbon Sources

Carbon Source	Growth	Carbon Source	Growth
Cellulose	+	Lactose	-
Grass	-	Maltose	-
Bark	+	Sucrose	+
Reed Canary Grass	+	Cane molasses	+
Corn Stover	-	Beet molasses	+
Cellulose Paper	-	Fructose	+
Starch	+	Lactate	+
Glucose	+	Succinate	-
Total Mixed Ration (TMR)	-	Glycerol	+
Xylose	-	Arabinose	-
Mannose	+	Ribose	-
Pectin	+	Mannitol	-
Molasses	+	Sorbitol	-
Cellobiose	+	No carbon	-
Gluconate	-		

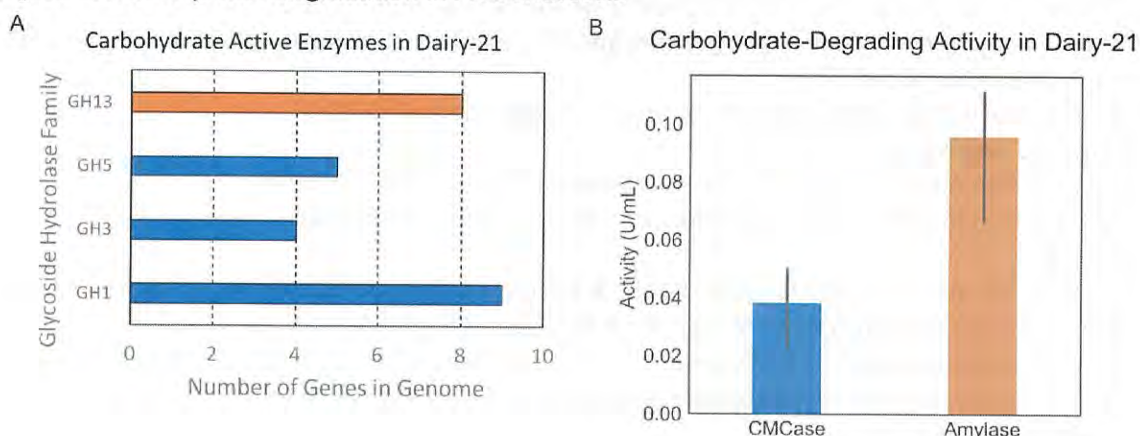
P. kudriavzevii was assessed for genes and activity against complex carbohydrates. As shown in Table 2 and Figure 1A, the genome contains genes necessary for degradation of starch (GH13) and cellulose (GH1, GH3, and GH5).

Table 2: Carbohydrate Active Enzymes in *P. kudriavzevii*

CAZy family	Number of genes	Pfam description	Pfam ID	Gene list
GH13	5	Alpha-amylase	PF00128	g3271.t1, g4521.t1, g4582.t1, g4815.t1, g4832.t1
GH5	5	Cellulase	PF00150	g452.t1, g695.t1, g2227.t1, g3550.t1, g3649.t1
GH3	4	Glyco_hydro_3	PF00933	g988.t1, g4731.t1, g4810.t1, g4983.t1
GH1	9	Glyco_hydro_1	PF00232	g4630.t1, g4736.t1, g4940.t1, g5040.t1, g5100.t1, g5257.t1, g5275.t1, g5369.t1, g5533.t1

In Figure 1B, the activities against starch (amylase activity) and against carboxymethylcellulose (CMCase activity) are displayed. 0.096 U/mL and 0.038 U/mL of amylase and CMCase activity were detected, repectively, where 1 Unit of activity is the amount of enzyme required to release 1 μmol of reducing sugar per minute.

Figure 1: Carbohydrate Degradation in *P. kudriavzevii*.



Conclusions

P. kudriavzevii is able to utilize multiple carbon sources for growth including monosaccharides, disaccharides, and polysaccharides. It contains the necessary genes for degradation of starch and cellulose, and activity against starch and carboxymethylcellulose (a soluble cellulose analog) were detected from the supernatant of *P. kudriavzevii* cultures. Taken together, these observations suggest that *P. kudriavzevii* plays a role in degradation of complex carbohydrates in its native rumen environment.

Documentation

The experimental design and results are located in Ascus Laboratory Notebook 31 and on the Ascus drive under /Ascus Biosciences/Cow/Digestibility_studies/

Signed: ______________________ Date: 11/18/19

References

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19: 455–477.

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Xiao, Z., Storms, R. & Tsang, A. (2005). Microplate-based carboxymethylcellulose assay for endoglucanase activity. Analytical biochemistry 342: 176-178

Objectives

The objective of this work was to determine the identity of *P. kudriavzevii* ASCUSDY21.

Methods

For ITS sequence analysis, the ITS 1 and 2 genes were amplified from *P. kudriavzevii* ASCUSDY21 using the ITS1F and ITS4 primers and sequenced using an Illumina Miseq. The resulting sequence was quality trimmed and compared to NCBI databases to establish the identity of the strain. The NCBI databases were queried on November 14, 2019.

For whole genome average nucleotide identity (ANI) analysis, genomic DNA was isolated via bead-based lysis using the MoBio PowerViral DNA kit (Carlsbad, CA). Sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA), and the resulting libraries were paired-end sequenced (2x300bp) on an Illumina Miseq. The draft genome was assembled using SPAdes [version 3.6.2] (Bankevich et al., 2012). MUMmer was used to generate the alignments for whole genome average nucleotide identity (ANI) (Kurtz et al., 2004).

Results

P. kudriavzevii ASCUSDY21 aligned with other isolates of *Pichia kudriavzevii*, with the top hits displayed in Table 1. The full list of hits and alignments can be seen in a separate file described in the documentation section.

Table 1: ITS analysis of *P. kudriavzevii* ASCUSDY21

Genus species (Accession)	Percent Match	Percent Coverage
Pichia kudriavzevii NT-108 (MN371886.1)	100%	100%
Pichia kudriavzevii XWB32-3 (MN310532.1)	100%	100%
Pichia kudriavzevii JYC563 (MN244404.1)	100%	100%

Whole genome ANI was used to confirm the ITS identification. The genome of three *P. kudriavzevii* isolates was accessed and assessed for ANI (% identity and coverage). As shown in Table 2, *P. kudriavzevii* ASCUSDY21 aligned with all three at an identity of 99% at a coverage of greater than 97% of the genome.

Table 2: Whole Genome ANI analysis of *P. kudriavzevii* ASCUSDY21

Organism (GenBank accession #)	Identity (%)	Coverage (%)
Pichia kudriavzevii CBS5147 (GCA_003054405)	99	98
Pichia kudriavzevii CBS573 (GCA_003054445)	99	98
Pichia kudriavzevii SJP (GCA_003033855)	99	97

Conclusions

P. kudriavzevii ASCUSDY21 was unambiguously identified as *Pichia kudriavzevii*, as it matched several *P. kudriavzevii* isolates at 100% over the ITS region sequenced and 99% ANI over 98% of the whole genome assembly.

Documentation

The full list of ITS hits and alignments can be found on the Ascus drive under (b) (4)

(b) (4)

The details of the ANI analysis can be found on the Ascus drive under (b) (4)

(b) (4)

(b) (4), (b) (6)

Signed: ________________ Date: 11/18/19

References

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19: 455–477.

Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, Salzberg SL. (2004) Versatile and open software for comparing large genomes. Genome Biol 5(2) :R12

RID: WV5W2122015
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Program: BLASTN
Database: nt Nucleotide collection (nt)
Query #1: Query ID: lcl|Query_40199 Length: 225

Sequences producing significant alignments:

Description	Max Score	Total Score	Query cover	E Value	Per. Ident	Accession

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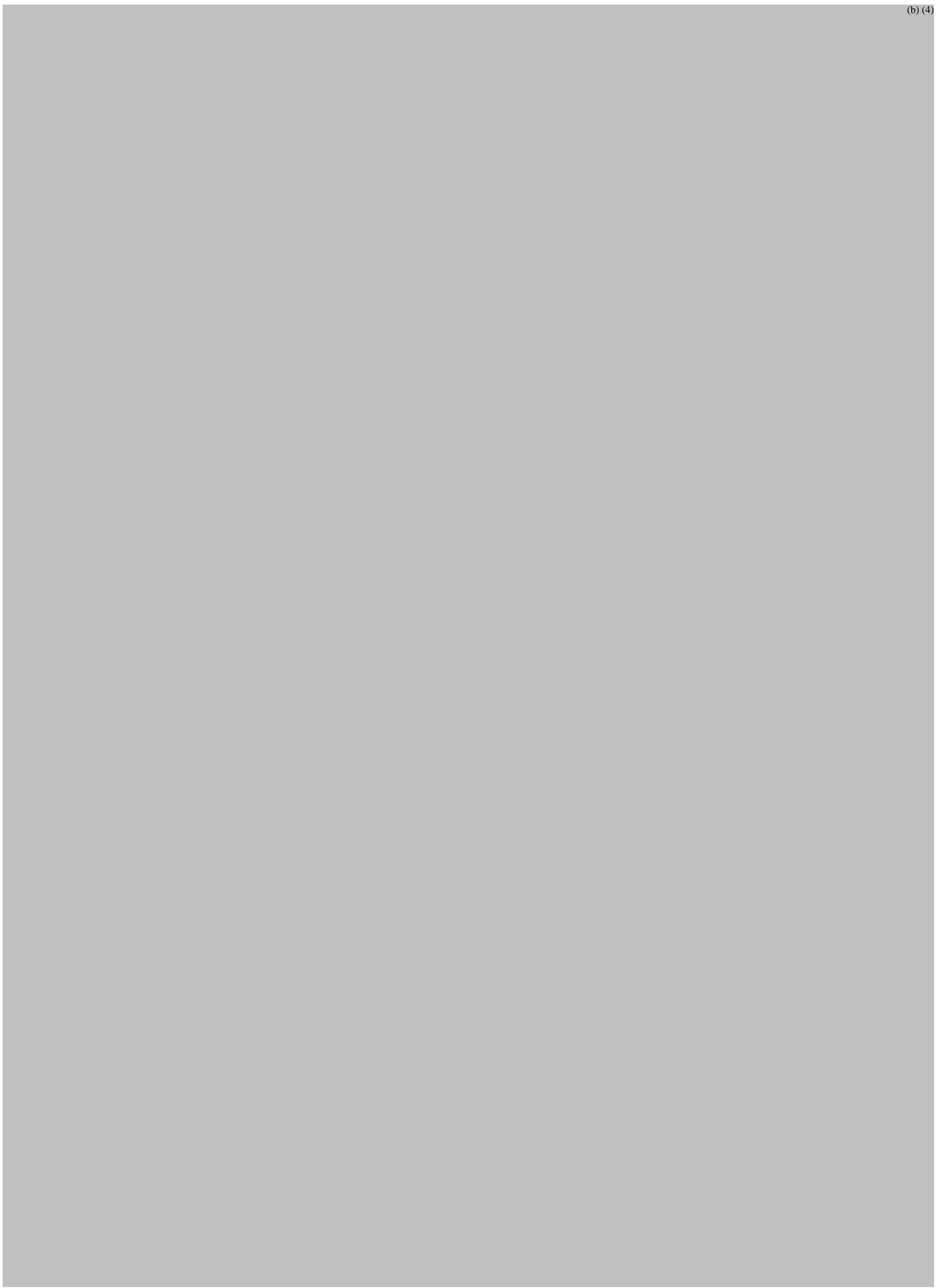

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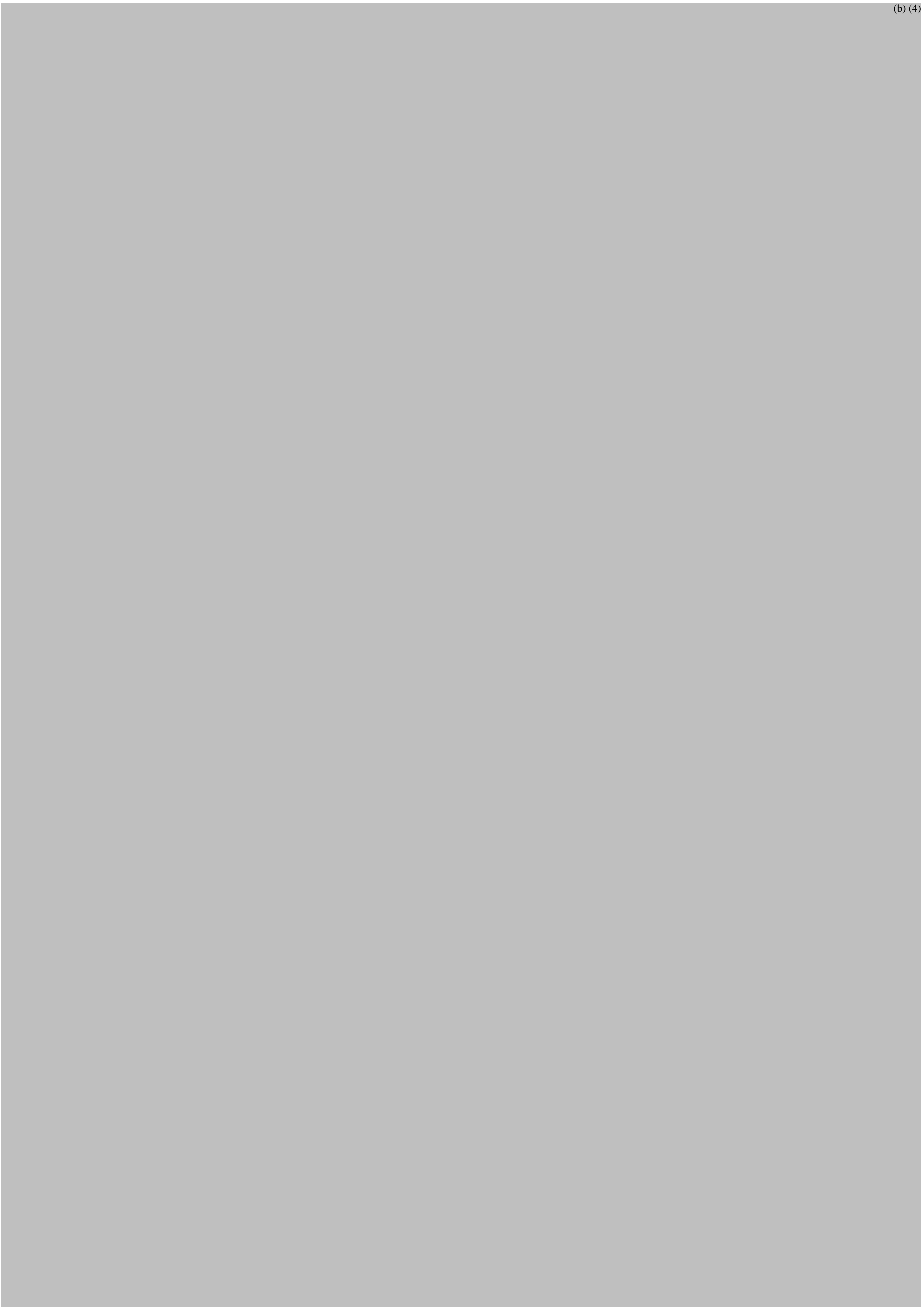
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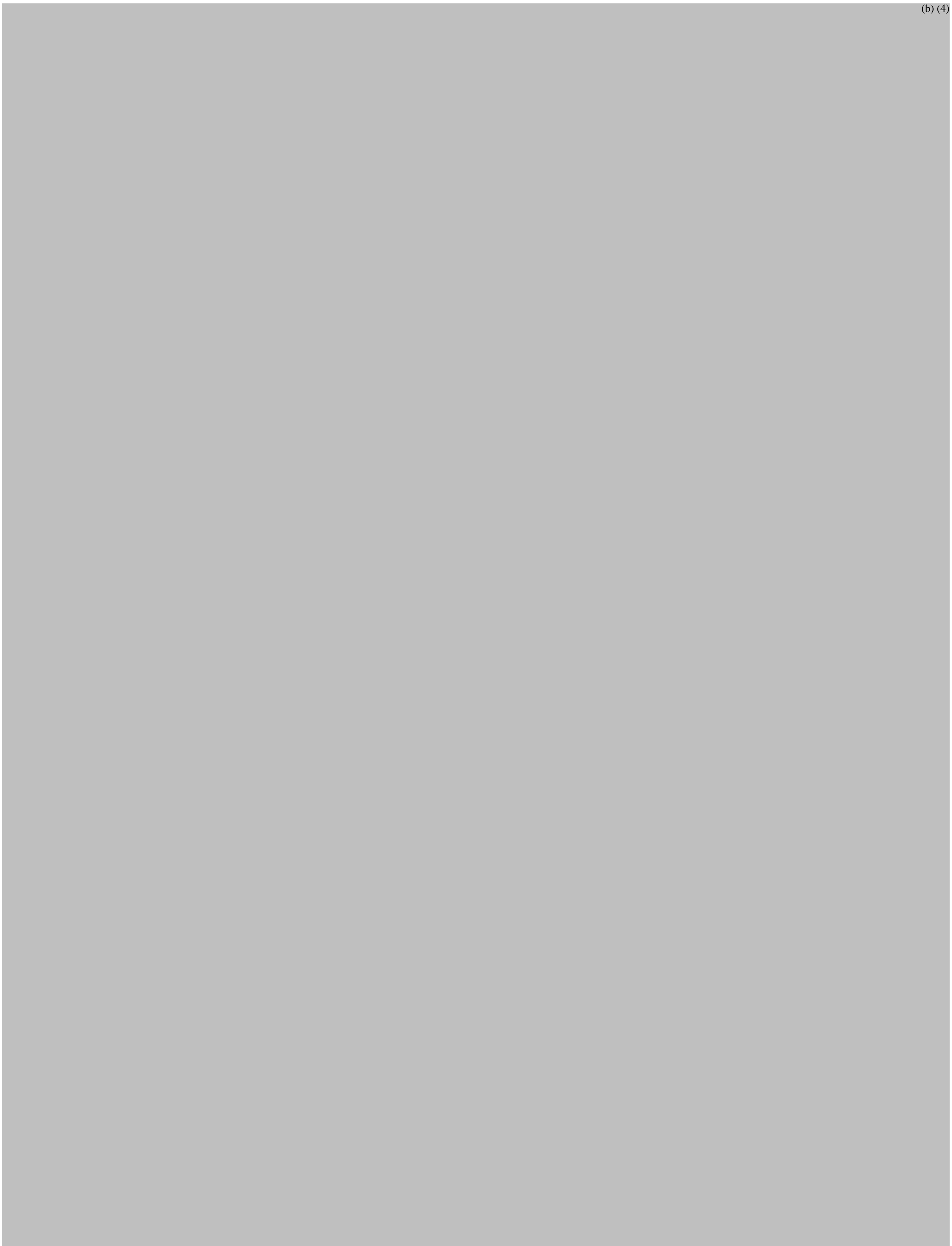
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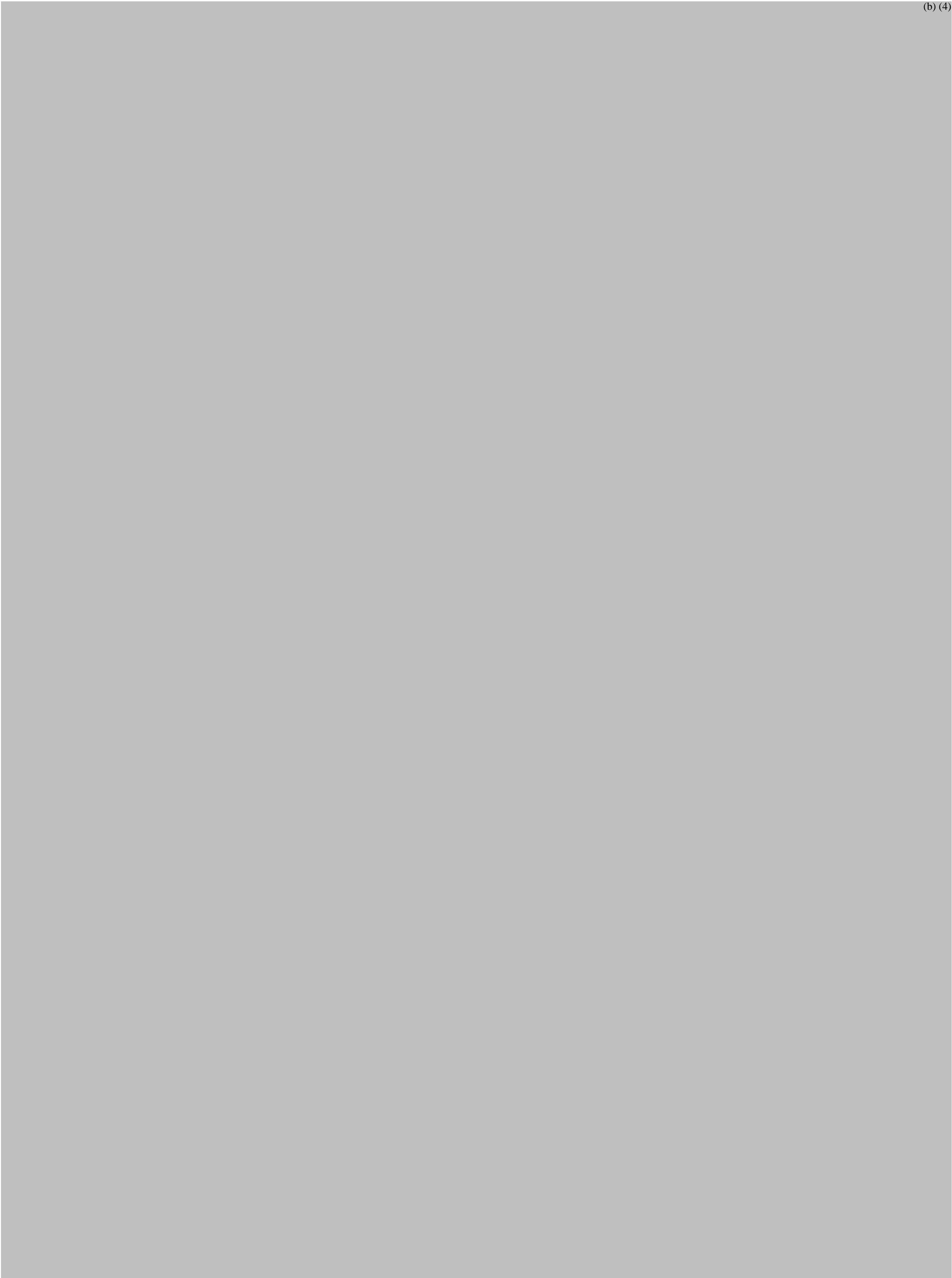

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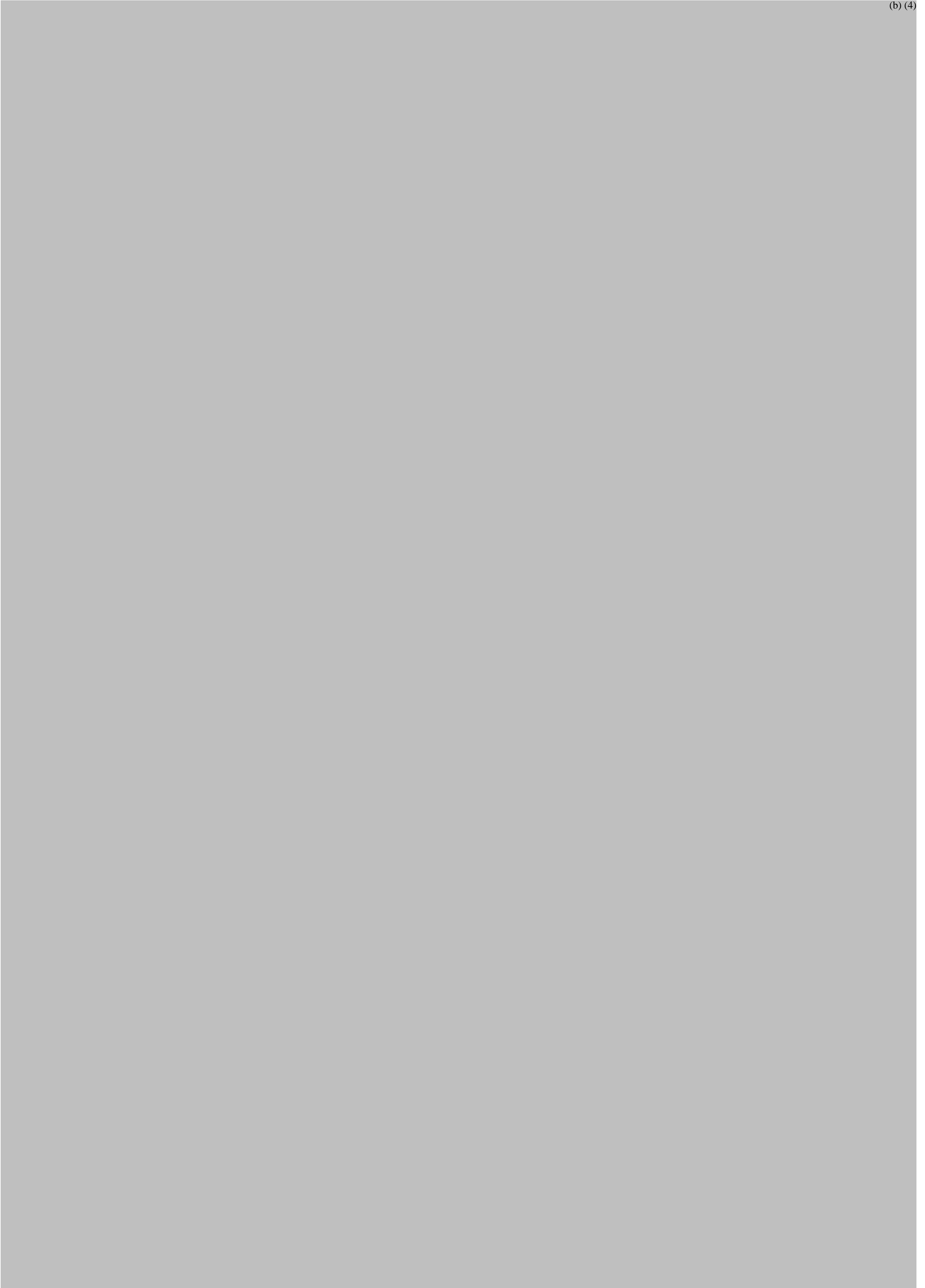
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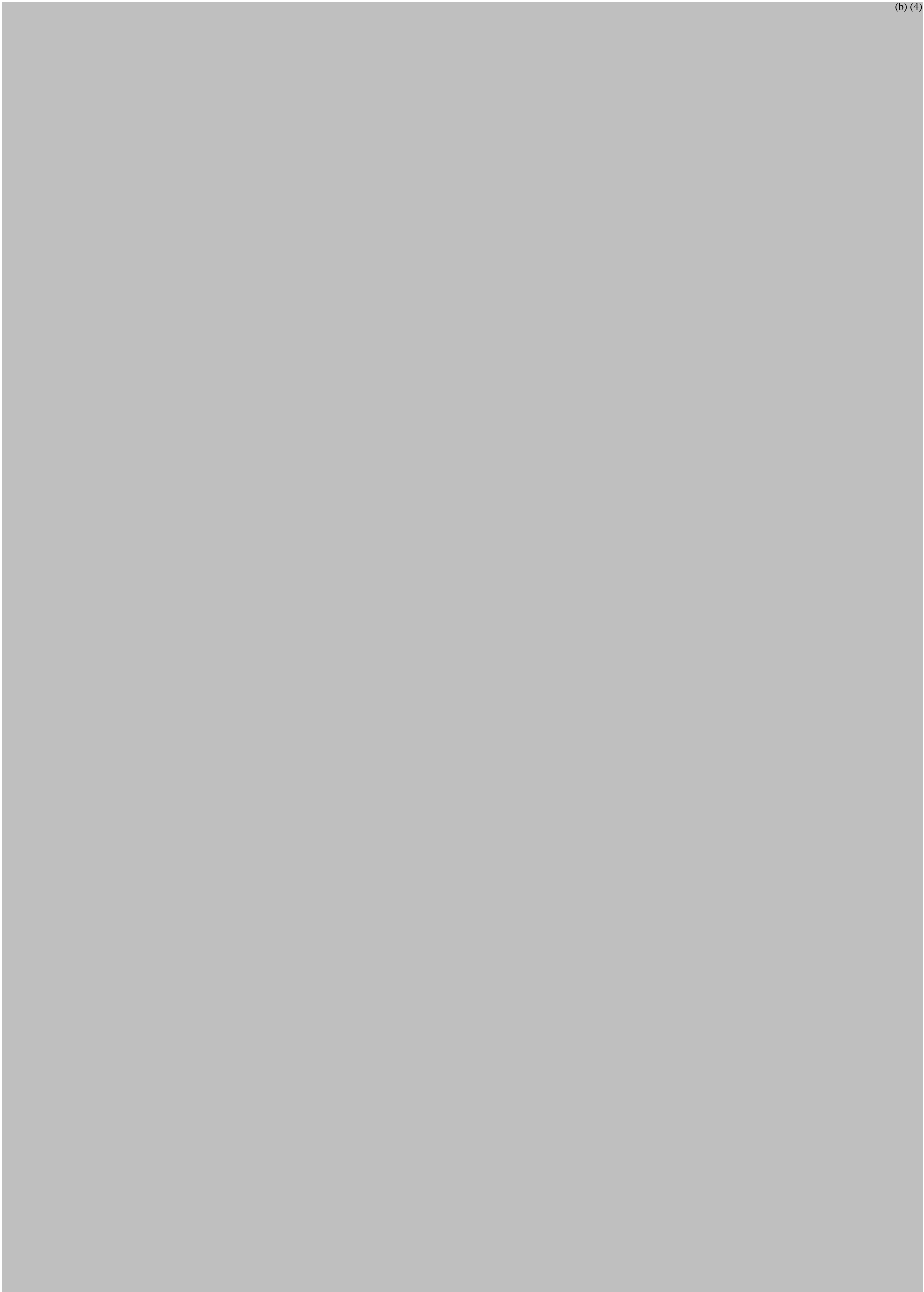
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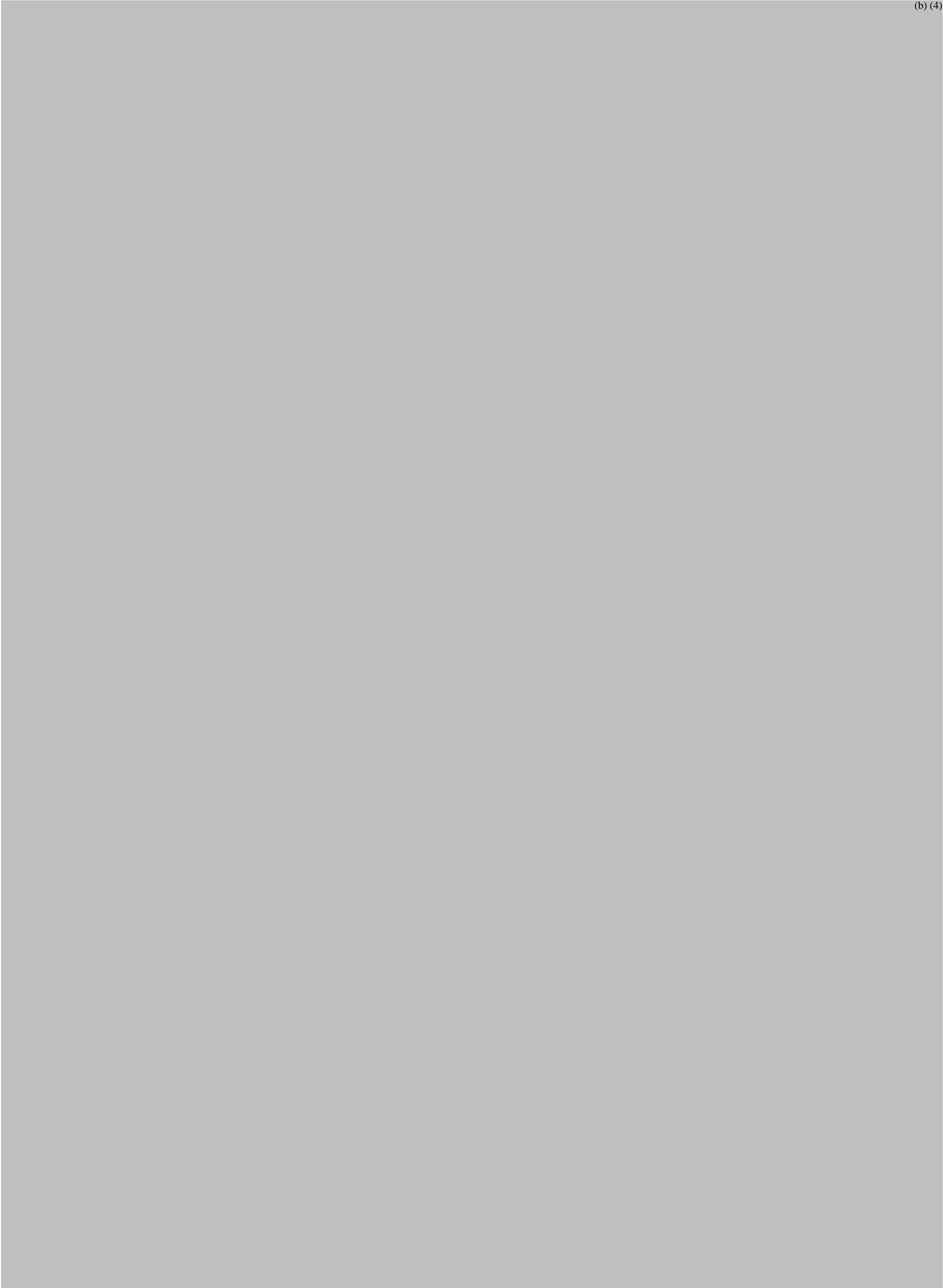
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FINAL REPORT

TITLE:	**Characterization of Ascus Biosciences Dairy-21: Susceptibility Profile**
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
CONDUCT DATES:	Receipt of isolate: October 26, 2017 MIC Testing: October 25, 2017 – November 3, 2017
SPONSOR:	Ascus Biosciences 6450 Lusk Blvd Suites E109/209 San Diego, CA 92121
INVESTIGATOR:	(b) (4)
VERSION:	FINAL
SIGNATURE:	(b) (4) 1/15/18 Date

Certified as an exact copy of the original by ____ Date Jan 15,2018

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Jan 15,2018

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Appendix

A **Protocol and SOP Documents**
Protocol
SOP-QC-45

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Jan 15,2018

1. OBJECTIVES

To determine the susceptibility profile of the *Pichia kudriavzevii* (Dairy-21) production strain to multiple antimicrobials.

2. STANDARDS OF COMPLIANCE

This study was conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs and to CLSI documents VET01 and M27 to the extent to which they were applicable as detailed in the protocol.

3. STUDY SITE

Susceptibility testing of the products was performed by (b) (4)

4. MATERIALS

The sponsor provided Dairy-21 production strain (i.e., *Pichia kudriavzevii*) was received on October 19, 2017. The culture was streaked to Sabouraud Dextrose Agar (SDA) to verify that the organism was viable, pure and morphologically typical of the purported species.

5. SUSCEPTIBILITY PROFILE

5.1. Procedure

The procedures listed in this protocol were written to comply with CLSI document M27-A3 entitled Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approve Standard – Third Edition with modification by the instruction sheet from the YeastOne panels (Thermo Scientific).

The isolate was tested using Sensititre Yeast One susceptibility panels with the concentrations of antifungals listed in Figure 2 of the Protocol.

(b) (4)

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Jan 15,2018

5.2. Media

(b) (4)

5.3. Incubation and Interpretation of Susceptibility Tests

(b) (4)

5.4. Quality Control

(b) (4) (Table 2).

(b) (4)

6. DISPOSITIONS

6.1. The MIC plates were discarded after their expiration.

6.2. The isolate, and all subcultures, were discarded after autoclaving. No retention culture was maintained.

7. RESULTS

MIC results of the *Pichia kudriavzevii* (Dairy-21) isolate are presented in Table 1. The isolate would be considered wild-type or susceptible according to both criteria (EUCAST and CLSI, as available) to micafungin, caspofungin, 5-flucytosine, voriconazole, itraconazole and fluconazole. The isolate would be considered susceptible to anidulafungin according to CLSI, but not EUCAST ECOFF values. According to EUCAST, the isolate would be non-wildtype against amphotericin B. No criteria were available for posaconazole, although the MIC was similar to voriconazole and itraconazole for which the isolate was considered susceptible.

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Jan 15,2018

Table 1. Listing of MIC of Antimicrobials and EFSA Microbiological Cut-off Values and EUCAST and CLSI Breakpoints for Bacteria [1]				
Antimicrobial	**Tested Range (µg/mL)**	**MIC (µg/mL) of the *Pichia kudriavzevii* (Dairy-21)**	**EUCAST ECOFF Values (µg/mL)**	**CLSI non-susceptible or resistant (µg/mL)**
Anidulafungin	0.015-8	(b) (4)	0.06	>2
Amphotericin B	0.12-8		1	Not Available
Micafungin	0.008-8		0.25	>2
Caspofungin	0.008-8		Not Available	>2
5-Flucytosine	0.06-64		Not Available	≥32
Posaconazole	0.008-8		Not Available	Not Available
Voriconazole	0.008-8		1	≥4
Itraconazole	0.015-16		1	≥1
Fluconazole	0.12-128		128	≥64

[1] EUCAST ECOFF values for *Candida krusei* (EUCAST) were accessed from https://mic.eucast.org/Eucast2/SearchController on 10/4/17 and CLSI M27-S3.

The MIC results of the quality control organisms were within the expected values as indicated in Table 2. The inoculum counts were within specifications.

Table 2. Quality Control Organism MIC Results				
	Candida parapsilosis ATCC 220197, (b) (4) code CR-1		*Issatchenkia orientalis Kudrjanzev* (ATCC 6258, MRI code IO-1)	
Antimicrobial	MIC	Acceptable Range[2]	MIC	Acceptable Range[2]
Anidulafungin	(b) (4)	0.25-2	(b) (4)	0.03-0.12
Amphotericin B		0.25-2		0.5-2
Micafungin		0.5-2		0.12-0.5
Caspofungin		0.25-1		0.12-1
5-Flucytosine		0.06-0.25		4-16
Posaconazole		0.06-0.25		0.06-0.5
Voriconazole		0.016-0.12		0.06-0.5
Itraconazole		0.12-0.5		0.12-1
Fluconazole		0.5-4		8-64

[2] Obtained from CLSI document M27-S3 - 24Hr

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Jan 15,2018

APPENDIX A. Protocol and SOP Documents

STUDY PROTOCOL

TITLE:	**Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of Antimicrobial Activity**
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
SPONSOR:	Ascus Biosciences 6450 Lusk Blvd Suites E109/209 San Diego, CA 92121
INVESTIGATOR:	(b) (4)
VERSION:	FINAL: October 10, 2017

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Jan 15,2018

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Certified as an exact copy of the original by ______ Date
Jan 15,2018

SIGNATURES

Sponsor Representative

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6450 Lusk Blvd
Suites E109/209
San Diego, CA 92121
Email: adam@ascusbiosciences.com
Tel. 707-601-2553

Signature Date

Investigator

(b) (4)

Certified as an exact copy of the original by ____ Date
Jan 15,2018

STUDY PROTOCOL No(s): (b) (4)
Version FINAL 10/10/17
Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of Antimicrobial Activity
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SIGNATURES

<u>Sponsor Representative</u>

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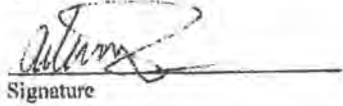
Email: adam@ascusbiosciences.com
Tel. 707-601-2553



Signature ______ Date 10/10/17

<u>Investigator</u>

Signature ______ Date ______

1. OBJECTIVES

1.1. To determine the Susceptibility Profile of the *Clostridium butyricum* (Dairy-20) and *Pichia kudriavzevii* (Dairy-21) production strains to multiple antimicrobials.

1.2. Determination of the antimicrobial properties of the *Clostridium butyricum* (Dairy-20) and *Pichia kudriavzevii* (Dairy-21) production strain supernatant.

2. STUDY TIMELINE

Anticipated study dates are:

Susceptibility Testing:	November 2017
Antimicrobial Properties:	November 2017

3. STANDARDS OF COMPLIANCE

This study will be conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs and to CLSI documents VET01, M11 and M27 to the extent to which they are applicable as detailed in this protocol. European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints or epidemiological cutoff values (ECOFFs) may be referenced for determining non-wildtype MIC values. Procedures for the susceptibility were designed to follow those in European Food Safety Authority (EFSA) *Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance*[4] as applicable and as detailed in this protocol.

4. STUDY SITE

Antimicrobial properties and susceptibility testing of the products will be performed by (b) (4).

5. MATERIALS AND METHODS

5.1. Isolates

The sponsor will provide the production strain and supernatant to test. The cultures will be streaked to an appropriate media (e.g., trypticase soy agar with 5% sheep blood agar (BA) for *Clostridium butyricum* and Sabouraud Dextrose Agar (SDA) for *Pichia kudriavzevii* to verify that the organisms are viable, pure and morphologically typical of the purported species.

[4] EFSA Journal 2012, 10(6): 2740

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5.2. Supernatants

The supernatants will be streaked onto BA or onto SDA as appropriate for the purported strain to verify their sterility. The BA will be incubated anaerobically at 36±2°C for 2 days prior to evaluation. The SDA will be incubated aerobically at 36±2°C for 2 days prior to evaluation. The supernatants will be sterilized by passing through a 0.45μm filter prior to continuing with testing if they are not sterile as provided by the Sponsor.

6. SUSCEPTIBILITY PROFILE

6.1. Procedure

Each production strain will be tested. Additional strains may be tested upon direction of the sponsor. The MIC values of the *Clostridium butyricum* will be compared to the "other Gram +" cut-off values published by EFSA or CLSI/EUCAST breakpoints to determine if a non-wild type strain (defined as potentially harboring resistance mechanisms) or if non-susceptible [refer to Table 3]. The cut-off/non-susceptible values for *Pichia kudriavzevii* will be evaluated in a similar manner comparing to values from EUCAST ECOFF values or CLSI interpretive criteria (Table 4).

The procedures listed in this protocol were written to comply with CLSI document M11-A8 entitled Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Eighth Edition for anaerobes using the broth microdilution procedure. The yeast isolates was tested according to CLSI document M27-A3 entitled Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approve Standard – Third Edition with modification by the instruction sheet from the YeastOne panels (Thermo Scientific).

MIC plates for anaerobes will be prepared by (b) (4)) with antimicrobials and doubling dilution concentrations as indicated in Figure 1. The yeast will be tested using Sensititre Yeast One susceptibility panels with the concentrations of antifungals listed in Figure 2. The isolates will be MIC tested according to SOP L-234 for anaerobes or as indicated below for yeast.

(b) (4)

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(b) (4)

6.2. Media

(b) (4)

6.3. Incubation and Interpretation of Susceptibility Tests

(b) (4)

6.4. Quality Control

(b) (4)

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Table 1. Quality Control Organisms and Acceptable MIC ranges for Anaerobes[2]

Antimicrobial	SBB	
	Bacteroides fragilis (Br-1) ATCC 25285	*Clostridium difficile* (CL-16) ATCC 700057
Ampicillin	2-8	1-4
Chloramphenicol	2-8	---
Clindamycin	0.5-2	2-8
Erythromycin	---	---
Gentamicin	---	---
Kanamycin	---	---
Streptomycin	---	---
Tetracycline	0.12-0.5	---
Vancomycin	---	0.5-4

[2]Obtained from CLSI document M100

Table 2. Quality Control Organisms and Acceptable MIC ranges for Yeast[3]

Antimicrobial	MIC values for RPMIG (µg/mL)	
	Candida parapsilosis ATCC 220197, (b) (4) code CR-1	*Issatchenkia orientalis Kudrjanzev* (ATCC 6258, (b) (4) code IO-1)
Anidulafungin	0.25-2	0.03-0.12
Amphotericin B	0.25-2	0.5-2
Micafungin	0.5-2	0.12-0.5
Caspofungin	0.25-1	0.12-1
5-Flucytosine	0.06-0.25	4-16
Posaconazole	0.06-0.25	0.06-0.5
Voriconazole	0.016-0.12	0.06-0.5
Itraconazole	0.12-0.5	0.12-1
Fluconazole	0.5-4	8-64

[3]Obtained from CLSI document M27-S3 - 24Hr

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Table 3. Listing of Antimicrobials and EFSA Microbiological Cut-off Values and EUCAST and CLSI Breakpoints for Bacteria [4]

		EFSA Microbiological Cut-off Values (µg/mL)	EUCAST Resistant Breakpoints (µg/mL)	CLSI Resistant Breakpoints (µg/mL)
Antimicrobial	Tested Range (µg/mL)	Other Gram +	Gram-positive anaerobes	Anaerobes
Ampicillin	0.06-64	1	8	≥2
Chloramphenicol	2-32	2	8	≥32
Clindamycin	0.03-32	0.25	4	≥8
Erythromycin	0.25-8	0.5	NA	NA
Gentamicin	0.12-32	4	NA	NA
Kanamycin	0.12-32	16	NA	NA
Streptomycin	0.12-32	0.5	NA	NA
Tetracycline	1-32	2	NA	≥16
Vancomycin	0.25-32	2	2	NA

[4] Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance; EFSA Journal 2012;10(6):2740. EUCAST breakpoints are for Gram-positive anaerobes, Clinical Breakpoint Tables V.7.1., CLSI M100S-26th Ed. Table 2J-1; NA=Not Available

Table 4. Listing of Antimicrobials and EUCAST ECOFF and CLSI Interpretive Criteria Values for Yeast[4]

Antimicrobial	Tested Range (µg/mL)	EUCAST ECOFF Values (µg/mL)	CLSI non-susceptible or resistant Interpretive Criteria (µg/mL)
Anidulafungin	0.015-8	0.06	>2
Amphotericin B	0.12-8	1	Not Available
Micafungin	0.008-8	0.25	>2
Caspofungin	0.008-8	Not Available	>2
5-Flucytosine	0.06-64	Not Available	≥32
Posaconazole	0.008-8	Not Available	Not Available
Voriconazole	0.008-8	1	≥4
Itraconazole	0.015-16	1	≥1
Fluconazole	0.12-128	128	≥64

[4] EUCAST ECOFF values for *Candida krusei* (EUCAST) were accessed from https://mic.eucast.org/Eucast2/SearchController on 10/4/17 and CLSI M27-S3.

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7. ABSENCE OF ANTIMICROBIAL PRODUCTION[5]

The presence of antimicrobial activity in the growth medium from both production strains (Dairy-20 and Dairy-21) will be tested. A portion of the growth medium from a typical production batch of bacteria, or a scaled down version, will be centrifuged and the supernatant will be sterile filtered (0.45µm) by the sponsor. The supernatant will be kept refrigerated (2-8°C) and shipped to (b) (4) for use within 20 days. A minimum of 5 mL will be provided to (b) (4). Stability of the product will not be determined. Additional supernatants may be tested as directed by the sponsor.

7.1. Preparation of Culture Plates

The following six organisms will be tested against each supernatant:

Organism	ATCC number	(b) (4) code	Dilution tested
Staphylococcus aureus	6538	Sta 11	1:10
Escherichia coli	11229	EC 96	1:10
Bacillus cereus	2	BC 5	1:10
Bacillus circulans	4516	Bi 1	1:10
Streptococcus pyogenes	12344	Str 59	1:20
Serratia marcescens	14041	SM 4	1:10

(b) (4)

[5] FAO (1999) Determination of Antibacterial Activity of enzyme preparations from the Combined Compendium of Food Additive Specifications, Vol. 4 (FAO/JECFA), pg 122.

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7.2. Disk Preparation

(b) (4)

7.3. Incubation

(b) (4)

7.4. Interpretation

(b) (4)

7.5. Quality Control

(b) (4)

8. RAW DATA, RECORDS, AND REPORTS

8.1. Data
All raw data will be recorded, handled, and stored according to facility SOPs, this protocol, and applicable regulatory requirements. All original data collected and

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Jan 15,2018

records generated in connection with the study will be archived at the study site. The following records will be maintained:

- Quality control records generated concurrent with all media and materials preparation, and lab testing,
- Protocols, protocol amendments, correspondence, reports and other documentation, including drafts of the final report
- Raw data and logs
- Documents related to any occurrence or situation that develops during the course of the trial that may affect the test results

All records will be maintained appropriately in labs and files as the project is ongoing, and thereafter in archives storage at (b) (4)

8.2. REPORTING OF RESULTS

A separate report will be issued for the production strain for each of the tests performed. Hence, a total of 4 reports will be issued according to the following Table:

	Report required (X) for the indicated test description for the Protocol section	
Production Strain	**1: Susceptibility Profile**	**2: Antimicrobial Activity**
Clostridium butyricum	X	X
Pichia kudriavzevii	X	X

If additional production strains are tested, reports will be issued in a similar manner, depending upon the tests required.

9. DISPOSITIONS

9.1. All surplus quantities of the provided supernatants will be discarded after autoclaving following report issue. No reserve samples will be maintained.

9.2. MIC plates will be discarded after their expiration.

9.3. Isolates will be discarded after autoclaving. No retention cultures will be maintained.

10. CHANGES TO PROTOCOL

Any change or revision to the approved protocol will be documented by written amendment that will be maintained with the protocol. As a minimum, the amendment will indicate the changes or revisions made, indicate the effective date, identify the protocol sections affected, explain the reasons for change and describe the impact on the study. The amendment will be

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Jan 15,2018

signed and dated by those who signed the protocol. Signatures will be obtained before implementation of the change if possible. If such is not possible, the investigator will attempt to obtain verbal prior authorization from the sponsor and follow with written documentation at the earliest opportunity. Protocol deviations are defined as unintended or unforeseeable necessary changes to the protocol. Protocol deviation reports list any action that is not/was not in accordance with the protocol. They must contain a detailed description of the deviation, its reason, and a description of its effect on the study.

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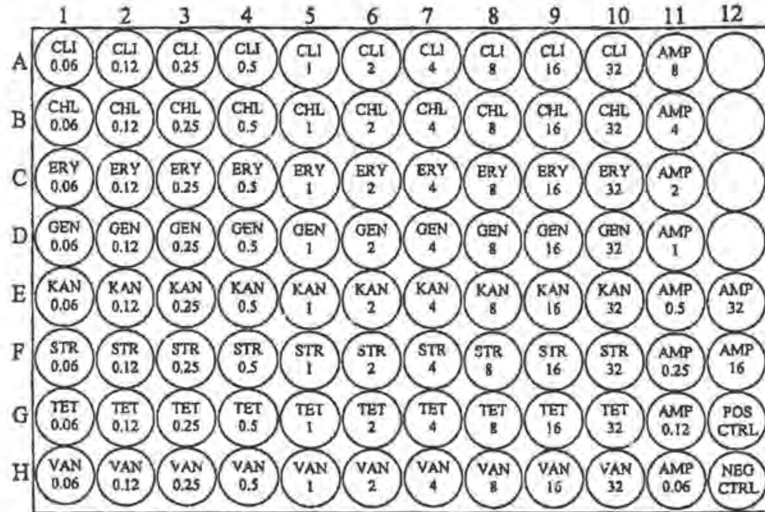
Figure 1. MIC Plate Diagram for (b) (4) Prepared Plate (1 isolate per plate)



Abbreviation	Antimicrobial		Abbreviation	Antimicrobial
AMP	Ampicillin		KAN	Kanamycin
CLI	Clindamycin		STR	Streptomycin
CHL	Chloramphenicol		TET	Tetracycline
ERY	Erythromycin		VAN	Vancomycin
GEN	Gentamicin			
POS CTRL	Inoculated, non-antimicrobial, positive control growth well		NEG CTRL	Uninoculated, non-antimicrobial, negative control well
(numerals indicate the concentration in µg/mL contained within the well)				

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Figure 2. MIC Plate Diagram for Yeast One Sensititre Plates (1 isolate per plate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	POS CTRL	AND 0.015	AND 0.03	AND 0.06	AND 0.12	AND 0.25	AND 0.5	AND 1	AND 2	AND 4	AND 8	AB 0.12
B	MF 0.008	MF 0.015	MF 0.03	MF 0.06	MF 0.12	MF 0.25	MF 0.5	MF 1	MF 2	MF 4	MF 8	AB 0.25
C	CAS 0.008	CAS 0.015	CAS 0.03	CAS 0.06	CAS 0.12	CAS 0.25	CAS 0.5	CAS 1	CAS 2	CAS 4	CAS 8	AB 0.5
D	FC 0.06	FC 0.12	FC 0.25	FC 0.5	FC 1	FC 2	FC 4	FC 8	FC 16	FC 32	FC 64	AB 1
E	PZ 0.008	PZ 0.015	PZ 0.03	PZ 0.06	PZ 0.12	PZ 0.25	PZ 0.5	PZ 1	PZ 2	PZ 4	PZ 8	AB 2
F	VOR 0.008	VOR 0.015	VOR 0.03	VOR 0.06	VOR 0.12	VOR 0.25	VOR 0.5	VOR 1	VOR 2	VOR 4	VOR 8	AB 4
G	IZ 0.015	IZ 0.03	IZ 0.06	IZ 0.12	IZ 0.25	IZ 0.5	IZ 1	IZ 2	IZ 4	IZ 8	IZ 16	AB 8
H	FZ 0.12	FZ 0.25	FZ 0.5	FZ 1	FZ 2	FZ 4	FZ 8	FZ 16	FZ 32	FZ 64	FZ 128	FZ 256

Abbreviation	Antimicrobial
AND	Anidulafungin
AB	Amphotericin B
MF	Micafungin
CAS	Caspofungin
FC	5-Flucytosine
POS CTRL	Inoculated, non-antimicrobial, positive control growth well

Abbreviation	Antimicrobial
PZ	Posaconazole
VOR	Voriconazole
IZ	Itraconazole
FZ	Fluconazole

(numerals indicate the concentration in µg/mL contained within the well)

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CONFIDENTIAL

ACCURACY VERIFICATION OF STANDARDIZED CULTURES
FOR SUSCEPTIBILITY TESTING

Section 1. General

Weekly, for each procedure described herein (when susceptibility tests are being conducted), after standardized and diluted test isolates have been inoculated according to the appropriate susceptibility procedure, the inoculum density will be tested to assure that the procedures for standardizing and diluting inoculum remain under control. Randomly select 5% of the susceptibility tests (up to five). One of the accuracy verification tests should be conducted on a QC organism in order to assist in determining the effect of an out-of-range colony count. If a study is performed in which no QC organisms are included alongside test isolates, the accuracy verification tests will be performed on test isolates only.

Calculations of the expected colony counts are based on the count of a 0.5 McFarland standard averaging 1.5×10^8 CFU/mL, and allow for errors associated with diluting and loss of viability occurring during diluting and plating. In general, the ranges for acceptable colony counts were developed to allow for ±1 LOG of target concentration.

A nutritive broth medium may be substituted for saline in any of the dilutions described in this SOP. For the more fastidious organisms, it is advisable to use a nutritive broth in lieu of saline (e.g., reduced Brucella broth [BB] or Brain Heart Infusion broth (BHI) should be used as the diluent for anaerobic bacteria).

Due to the difficulty of maintaining anaerobiosis while handling anaerobic organisms during the dilution process, and the slow growth patterns of anaerobic organisms, the resulting counts may be inaccurate. These counts will be recorded but will not be used to accept or reject the results of the susceptibility testing. If anaerobic organism counts are consistently low, the dilution procedure may be changed to account for loss of viability by handling the organisms during the inoculation procedure.

Tests should be conducted within 10 minutes of performing the susceptibility procedure.

Section 2. Agar Dilution

A. General:

(b) (4)

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ACCURACY VERIFICATION OF STANDARDIZED CULTURES
FOR SUSCEPTIBILITY TESTING

B. Anaerobic Bacteria:

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Revision #9
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ACCURACY VERIFICATION OF STANDARDIZED CULTURES
FOR SUSCEPTIBILITY TESTING

(b) (4)

Section 3. Disk Diffusion and/or E Test

(b) (4)

Section 4. Microdilution (b) (4) prepared MIC plates and/or Sensititre)

A. Bacteria

(b) (4)

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ACCURACY VERIFICATION OF STANDARDIZED CULTURES
FOR SUSCEPTIBILITY TESTING

B. Mycoplasma

C. Anaerobic Bacteria

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ACCURACY VERIFICATION OF STANDARDIZED CULTURES FOR SUSCEPTIBILITY TESTING

Section 5. Interpretation of Results

If QC organism and test isolates fall within the acceptable range, data will be recorded.

If any counts fall out of the ranges described in Sections 2, 3 or 4, the Laboratory Director should be notified to determine if the low or high count is severe enough to have an effect on test isolates.

If results consistently show counts to be unacceptable, the following should be checked to determine cause and the corrective action to take:

- McFarland Standard quality: Prepare fresh standards if needed.
- Are test isolates being properly compared to McFarland standard? Re-train personnel if needed.
- Proper culture dilution: Re-train personnel if needed, and/or adjust dilution procedure.
- Pipettor set to the correct volume, adjust if necessary.
- Is the proper dilution and plating procedure being followed as described in this SOP? Re-train personnel if needed.

If counts for a particular organism are consistently out of range, the dilution procedure for preparation of inoculum of that particular organism should be adjusted after determining actual counts of a 0.5 McFarland standard adjusted suspension of that organism.

Prepared by: (b) (4)
Date: 5/31/16

Reviewed by: (b) (4)
Date: 5/31/16

Approved by: (b) (4)
Date: 5/31/16
Effective Date: 6/14/16

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(b) (4)

FINAL REPORT

TITLE:	**Characterization of Ascus Biosciences Dairy-21: Antibacterial Properties**
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
CONDUCT DATES:	Receipt of supernatant: November 14, 2017 Testing of supernatant: November 15, 2017 – November 17, 2017
SPONSOR:	Ascus Biosciences 6450 Lusk Blvd Suites E109/209 San Diego, CA 92121
INVESTIGATOR:	(b) (4)
VERSION:	FINAL

SIGNATURE: (b) (4)

Principal Investigator

1/15/18
Date

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Jan 15,2018

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Jan 15,2018

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OBJECTIVES

To determine the antimicrobial properties of the *Pichia kudriavzevii* (Dairy-21) production strain supernatant.

STANDARDS OF COMPLIANCE

This study was conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs as detailed in the protocol.

STUDY SITE

Antimicrobial property testing of the product was performed by (b) (4)

MATERIALS

The sponsor provided Dairy-21 supernatant (Lot AS110617f4) was prepared by centrifugation at 25,000RPM for 15 minutes followed by sterile filtration with a 0.2um membrane. The sample was received on November 14, 2017.

ANTIMICROBIAL PROPERTIES

A portion of the growth medium from a typical production batch of bacteria, or a scaled down version, was centrifuged and the supernatant sterile filtered by the sponsor. The supernatant was kept refrigerated (2-8°C) and shipped to (b) (4) and used 9 days after preparation.

1.1. Preparation of Culture Plates

The following six organisms were tested against the supernatant:

Organism	ATCC number	(b) (4) code	Dilution tested
Staphylococcus aureus	6538	Sta 11	1:10
Escherichia coli	11229	EC 96	1:10
Bacillus cereus	2	BC 5	1:10
Bacillus circulans	4516	Bi 1	1:10
Streptococcus pyogenes	12344	Str 59	1:20
Serratia marcescens	14041	SM 4	1:10

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1.2. Disk Preparation

(b) (4)

1.3. Incubation

(b) (4)

1.4. Interpretation

(b) (4)

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1.5. Quality Control

(b) (4)

DISPOSITIONS

The supernatant was discarded after autoclaving and issue of the final report. No retention sample was maintained.

RESULTS

No zones of inhibition were observed for Dairy-21 supernatant lot, or the sterile distilled water control. A zone of inhibition was observed for the enrofloxacin positive control for each organism as indicated in the table below:

Table 1. Zone Diameters from Dairy-21 Supernatant and Controls

			Zone Diameter for the indicated solution (mm)		
Organism	ATCC number	(b) (4) code	Dairy-21 Lot: AS110617f4	Sterile Distilled water	Enrofloxacin
Staphylococcus aureus	6538	Sta 11	(b) (4)	≤12.7	(b) (4)
Escherichia coli	11229	EC 96		≤12.7	
Bacillus cereus	2	BC 5		≤12.7	
Bacillus circulans	4516	Bi 1		≤12.7	
Streptococcus pyogenes	12344	Str 59		≤12.7	
Serratia marcescens	14041	SM 4		≤12.7	

Following incubation, pictures were taken of each organism seeded into the agar onto which a saturated disk of supernatant and controls were placed according to the protocol. These pictures are included in Appendix B. No zones of inhibition are observed in these pictures.

CONCLUSION

The Dairy-21 supernatant (Lot AS110617f4) exhibited no antibacterial activity against the 6 strains representative of Gram positive and Gram negative bacteria.

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APPENDIX A. Protocol

STUDY PROTOCOL

TITLE:	**Characterization of Ascus Biosciences Dairy-20 and Dairy-21: Susceptibility Profile and Absence of Antimicrobial Activity**
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
SPONSOR:	Ascus Biosciences 6450 Lusk Blvd Suites E109/209 San Diego, CA 92121
INVESTIGATOR:	(b) (4)
VERSION:	FINAL: October 10, 2017

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Jan 15,2018

SIGNATURES

Sponsor Representative

Adam Taylor
Ascus Biosciences
6450 Lusk Blvd
Suites E109/209
San Diego, CA 92121
Email: adam@ascusbiosciences.com
Tel. 707-601-2553

Signature ______ Date ______

Investigator

(b) (4)

(b) (4)

Signature ______ Date 10/10/17

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Jan 15,2018

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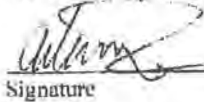
10/10/17

Signature   Date

Investigator

(b) (4)

Signature   Date

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1. OBJECTIVES

1.1. To determine the Susceptibility Profile of the *Clostridium butyricum* (Dairy-20) and *Pichia kudriavzevii* (Dairy-21) production strains to multiple antimicrobials.

1.2. Determination of the antimicrobial properties of the *Clostridium butyricum* (Dairy-20) and *Pichia kudriavzevii* (Dairy-21) production strain supernatant.

2. STUDY TIMELINE

Anticipated study dates are:

Susceptibility Testing:	November 2017
Antimicrobial Properties:	November 2017

3. STANDARDS OF COMPLIANCE

This study will be conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs and to CLSI documents VET01, M11 and M27 to the extent to which they are applicable as detailed in this protocol. European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints or epidemiological cutoff values (ECOFFs) may be referenced for determining non-wildtype MIC values. Procedures for the susceptibility were designed to follow those in European Food Safety Authority (EFSA) *Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance*[4] as applicable and as detailed in this protocol.

4. STUDY SITE

Antimicrobial properties and susceptibility testing of the products will be performed by (b) (4).

5. MATERIALS AND METHODS

5.1. Isolates

The sponsor will provide the production strain and supernatant to test. The cultures will be streaked to an appropriate media (e.g., trypticase soy agar with 5% sheep blood agar (BA) for *Clostridium butyricum* and Sabouraud Dextrose Agar (SDA) for *Pichia kudriavzevii* to verify that the organisms are viable, pure and morphologically typical of the purported species.

[4] EFSA Journal 2012, 10(6): 2740

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5.2. Supernatants

The supernatants will be streaked onto BA or onto SDA as appropriate for the purported strain to verify their sterility. The BA will be incubated anaerobically at 36±2°C for 2 days prior to evaluation. The SDA will be incubated aerobically at 36±2°C for 2 days prior to evaluation. The supernatants will be sterilized by passing through a 0.45µm filter prior to continuing with testing if they are not sterile as provided by the Sponsor.

6. SUSCEPTIBILITY PROFILE

6.1. Procedure

Each production strain will be tested. Additional strains may be tested upon direction of the sponsor. The MIC values of the *Clostridium butyricum* will be compared to the "other Gram +" cut-off values published by EFSA or CLSI/EUCAST breakpoints to determine if a non-wild type strain (defined as potentially harboring resistance mechanisms) or if non-susceptible [refer to Table 3]. The cut-off/non-susceptible values for *Pichia kudriavzevii* will be evaluated in a similar manner comparing to values from EUCAST ECOFF values or CLSI interpretive criteria (Table 4).

The procedures listed in this protocol were written to comply with CLSI document M11-A8 entitled Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Eighth Edition for anaerobes using the broth microdilution procedure. The yeast isolates was tested according to CLSI document M27-A3 entitled Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approve Standard – Third Edition with modification by the instruction sheet from the YeastOne panels (Thermo Scientific).

MIC plates for anaerobes will be prepared by (b) (4) with antimicrobials and doubling dilution concentrations as indicated in Figure 1. The yeast will be tested using Sensititre Yeast One susceptibility panels with the concentrations of antifungals listed in Figure 2. The isolates will be MIC tested according to SOP L-234 for anaerobes or as indicated below for yeast.

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(b) (4)

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Table 1. Quality Control Organisms and Acceptable MIC ranges for Anaerobes[2]

	SBB	
Antimicrobial	*Bacteroides fragilis* (Br-1) ATCC 25285	*Clostridium difficile* (CL-16) ATCC 700057
Ampicillin	2-8	1-4
Chloramphenicol	2-8	---
Clindamycin	0.5-2	2-8
Erythromycin	---	---
Gentamicin	---	---
Kanamycin	---	---
Streptomycin	---	---
Tetracycline	0.12-0.5	---
Vancomycin	---	0.5-4

[2]Obtained from CLSI document M100

Table 2. Quality Control Organisms and Acceptable MIC ranges for Yeast[3]

	MIC values for RPMIG (µg/mL)	
Antimicrobial	*Candida parapsilosis* ATCC 220197, (b) (4) code CR-1	*Issatchenkia orientalis Kudrjanzev* (ATCC 6258, (b) (4) code IO-1)
Anidulafungin	0.25-2	0.03-0.12
Amphotericin B	0.25-2	0.5-2
Micafungin	0.5-2	0.12-0.5
Caspofungin	0.25-1	0.12-1
5-Flucytosine	0.06-0.25	4-16
Posaconazole	0.06-0.25	0.06-0.5
Voriconazole	0.016-0.12	0.06-0.5
Itraconazole	0.12-0.5	0.12-1
Fluconazole	0.5-4	8-64

[3]Obtained from CLSI document M27-S3 - 24Hr

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Table 3. Listing of Antimicrobials and EFSA Microbiological Cut-off Values and EUCAST and CLSI Breakpoints for Bacteria [4]

		EFSA Microbiological Cut-off Values (µg/mL)	EUCAST Resistant Breakpoints (µg/mL)	CLSI Resistant Breakpoints (µg/mL)
Antimicrobial	**Tested Range (µg/mL)**	Other Gram +	Gram-positive anaerobes	Anaerobes
Ampicillin	0.06-64	1	8	≥2
Chloramphenicol	2-32	2	8	≥32
Clindamycin	0.03-32	0.25	4	≥8
Erythromycin	0.25-8	0.5	NA	NA
Gentamicin	0.12-32	4	NA	NA
Kanamycin	0.12-32	16	NA	NA
Streptomycin	0.12-32	0.5	NA	NA
Tetracycline	1-32	2	NA	≥16
Vancomycin	0.25-32	2	2	NA

[4] Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance; EFSA Journal 2012;10(6):2740. EUCAST breakpoints are for Gram-positive anaerobes, Clinical Breakpoint Tables V.7.1., CLSI M100S-26th Ed. Table 2J-1; NA=Not Available

Table 4. Listing of Antimicrobials and EUCAST ECOFF and CLSI Interpretive Criteria Values for Yeast[4]

Antimicrobial	Tested Range (µg/mL)	EUCAST ECOFF Values (µg/mL)	CLSI non-susceptible or resistant Interpretive Criteria (µg/mL)
Anidulafungin	0.015-8	0.06	>2
Amphotericin B	0.12-8	1	Not Available
Micafungin	0.008-8	0.25	>2
Caspofungin	0.008-8	Not Available	>2
5-Flucytosine	0.06-64	Not Available	≥32
Posaconazole	0.008-8	Not Available	Not Available
Voriconazole	0.008-8	1	≥4
Itraconazole	0.015-16	1	≥1
Fluconazole	0.12-128	128	≥64

[4] EUCAST ECOFF values for *Candida krusei* (EUCAST) were accessed from https://mic.eucast.org/Eucast2/SearchController on 10/4/17 and CLSI M27-S3.

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7. ABSENCE OF ANTIMICROBIAL PRODUCTION[5]

The presence of antimicrobial activity in the growth medium from both production strains (Dairy-20 and Dairy-21) will be tested. A portion of the growth medium from a typical production batch of bacteria, or a scaled down version, will be centrifuged and the supernatant will be sterile filtered (0.45µm) by the sponsor. The supernatant will be kept refrigerated (2-8°C) and shipped to (b) (4) for use within 20 days. A minimum of 5 mL will be provided to (b) (4). Stability of the product will not be determined. Additional supernatants may be tested as directed by the sponsor.

7.1. Preparation of Culture Plates

The following six organisms will be tested against each supernatant:

Organism	ATCC number	(b) (4) code	Dilution tested
Staphylococcus aureus	6538	Sta 11	1:10
Escherichia coli	11229	EC 96	1:10
Bacillus cereus	2	BC 5	1:10
Bacillus circulans	4516	Bi 1	1:10
Streptococcus pyogenes	12344	Str 59	1:20
Serratia marcescens	14041	SM 4	1:10

[5] FAO (1999) Determination of Antibacterial Activity of enzyme preparations from the Combined Compendium of Food Additive Specifications, Vol. 4 (FAO/JECFA), pg 122.

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7.2. Disk Preparation

(b) (4)

7.3. Incubation

(b) (4)

7.4. Interpretation

(b) (4)

7.5. Quality Control

(b) (4)

8. RAW DATA, RECORDS, AND REPORTS

8.1. Data

All raw data will be recorded, handled, and stored according to facility SOPs, this protocol, and applicable regulatory requirements. All original data collected and

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records generated in connection with the study will be archived at the study site. The following records will be maintained:

- Quality control records generated concurrent with all media and materials preparation, and lab testing,
- Protocols, protocol amendments, correspondence, reports and other documentation, including drafts of the final report
- Raw data and logs
- Documents related to any occurrence or situation that develops during the course of the trial that may affect the test results

All records will be maintained appropriately in labs and files as the project is ongoing, and thereafter in archives storage at (b) (4).

8.2. REPORTING OF RESULTS

A separate report will be issued for the production strain for each of the tests performed. Hence, a total of 4 reports will be issued according to the following Table:

Production Strain	Report required (X) for the indicated test description for the Protocol section	
	1: Susceptibility Profile	2: Antimicrobial Activity
Clostridium butyricum	X	X
Pichia kudrtavzevii	X	X

If additional production strains are tested, reports will be issued in a similar manner, depending upon the tests required.

9. DISPOSITIONS

9.1. All surplus quantities of the provided supernatants will be discarded after autoclaving following report issue. No reserve samples will be maintained.

9.2. MIC plates will be discarded after their expiration.

9.3. Isolates will be discarded after autoclaving. No retention cultures will be maintained.

10. CHANGES TO PROTOCOL

Any change or revision to the approved protocol will be documented by written amendment that will be maintained with the protocol. As a minimum, the amendment will indicate the changes or revisions made, indicate the effective date, identify the protocol sections affected, explain the reasons for change and describe the impact on the study. The amendment will be

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signed and dated by those who signed the protocol. Signatures will be obtained before implementation of the change if possible. If such is not possible, the investigator will attempt to obtain verbal prior authorization from the sponsor and follow with written documentation at the earliest opportunity. Protocol deviations are defined as unintended or unforeseeable necessary changes to the protocol. Protocol deviation reports list any action that is not/was not in accordance with the protocol. They must contain a detailed description of the deviation, its reason, and a description of its effect on the study.

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Figure 1. MIC Plate Diagram for (b) (4) Prepared Plate (1 isolate per plate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CLI 0.06	CLI 0.12	CLI 0.25	CLI 0.5	CLI 1	CLI 2	CLI 4	CLI 8	CLI 16	CLI 32	AMP 8	
B	CHL 0.06	CHL 0.12	CHL 0.25	CHL 0.5	CHL 1	CHL 2	CHL 4	CHL 8	CHL 16	CHL 32	AMP 4	
C	ERY 0.06	ERY 0.12	ERY 0.25	ERY 0.5	ERY 1	ERY 2	ERY 4	ERY 8	ERY 16	ERY 32	AMP 2	
D	GEN 0.06	GEN 0.12	GEN 0.25	GEN 0.5	GEN 1	GEN 2	GEN 4	GEN 8	GEN 16	GEN 32	AMP 1	
E	KAN 0.06	KAN 0.12	KAN 0.25	KAN 0.5	KAN 1	KAN 2	KAN 4	KAN 8	KAN 16	KAN 32	AMP 0.5	AMP 32
F	STR 0.06	STR 0.12	STR 0.25	STR 0.5	STR 1	STR 2	STR 4	STR 8	STR 16	STR 32	AMP 0.25	AMP 16
G	TET 0.06	TET 0.12	TET 0.25	TET 0.5	TET 1	TET 2	TET 4	TET 8	TET 16	TET 32	AMP 0.12	POS CTRL
H	VAN 0.06	VAN 0.12	VAN 0.25	VAN 0.5	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	AMP 0.06	NEG CTRL

Abbreviation	Antimicrobial		Abbreviation	Antimicrobial
AMP	Ampicillin		KAN	Kanamycin
CLI	Clindamycin		STR	Streptomycin
CHL	Chloramphenicol		TET	Tetracycline
ERY	Erythromycin		VAN	Vancomycin
GEN	Gentamicin			
POS CTRL	Inoculated, non-antimicrobial, positive control growth well		NEG CTRL	Uninoculated, non-antimicrobial, negative control well
(numerals indicate the concentration in µg/mL contained within the well)				

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Figure 2. MIC Plate Diagram for Yeast One Sensititre Plates (1 isolate per plate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	POS CTRL	AND 0.015	AND 0.03	AND 0.06	AND 0.12	AND 0.25	AND 0.5	AND 1	AND 2	AND 4	AND 8	AB 0.12
B	MF 0.008	MF 0.015	MF 0.03	MF 0.06	MF 0.12	MF 0.25	MF 0.5	MF 1	MF 2	MF 4	MF 8	AB 0.25
C	CAS 0.008	CAS 0.015	CAS 0.03	CAS 0.06	CAS 0.12	CAS 0.25	CAS 0.5	CAS 1	CAS 2	CAS 4	CAS 8	AB 0.5
D	FC 0.06	FC 0.12	FC 0.25	FC 0.5	FC 1	FC 2	FC 4	FC 8	FC 16	FC 32	FC 64	AB 1
E	PZ 0.008	PZ 0.015	PZ 0.03	PZ 0.06	PZ 0.12	PZ 0.25	PZ 0.5	PZ 1	PZ 2	PZ 4	PZ 8	AB 2
F	VOR 0.008	VOR 0.015	VOR 0.03	VOR 0.06	VOR 0.12	VOR 0.25	VOR 0.5	VOR 1	VOR 2	VOR 4	VOR 8	AB 4
G	IZ 0.015	IZ 0.03	IZ 0.06	IZ 0.12	IZ 0.25	IZ 0.5	IZ 1	IZ 2	IZ 4	IZ 8	IZ 16	AB 8
H	FZ 0.12	FZ 0.25	FZ 0.5	FZ 1	FZ 2	FZ 4	FZ 8	FZ 16	FZ 32	FZ 64	FZ 128	FZ 256

Abbreviation	Antimicrobial	Abbreviation	Antimicrobial
AND	Anidulafungin	PZ	Posaconazole
AB	Amphotericin B	VOR	Voriconazole
MF	Micafungin	IZ	Itraconazole
CAS	Caspofungin	FZ	Fluconazole
FC	5-Flucytosine		
POS CTRL	Inoculated, non-antimicrobial, positive control growth well		
(numerals indicate the concentration in µg/mL contained within the well)			

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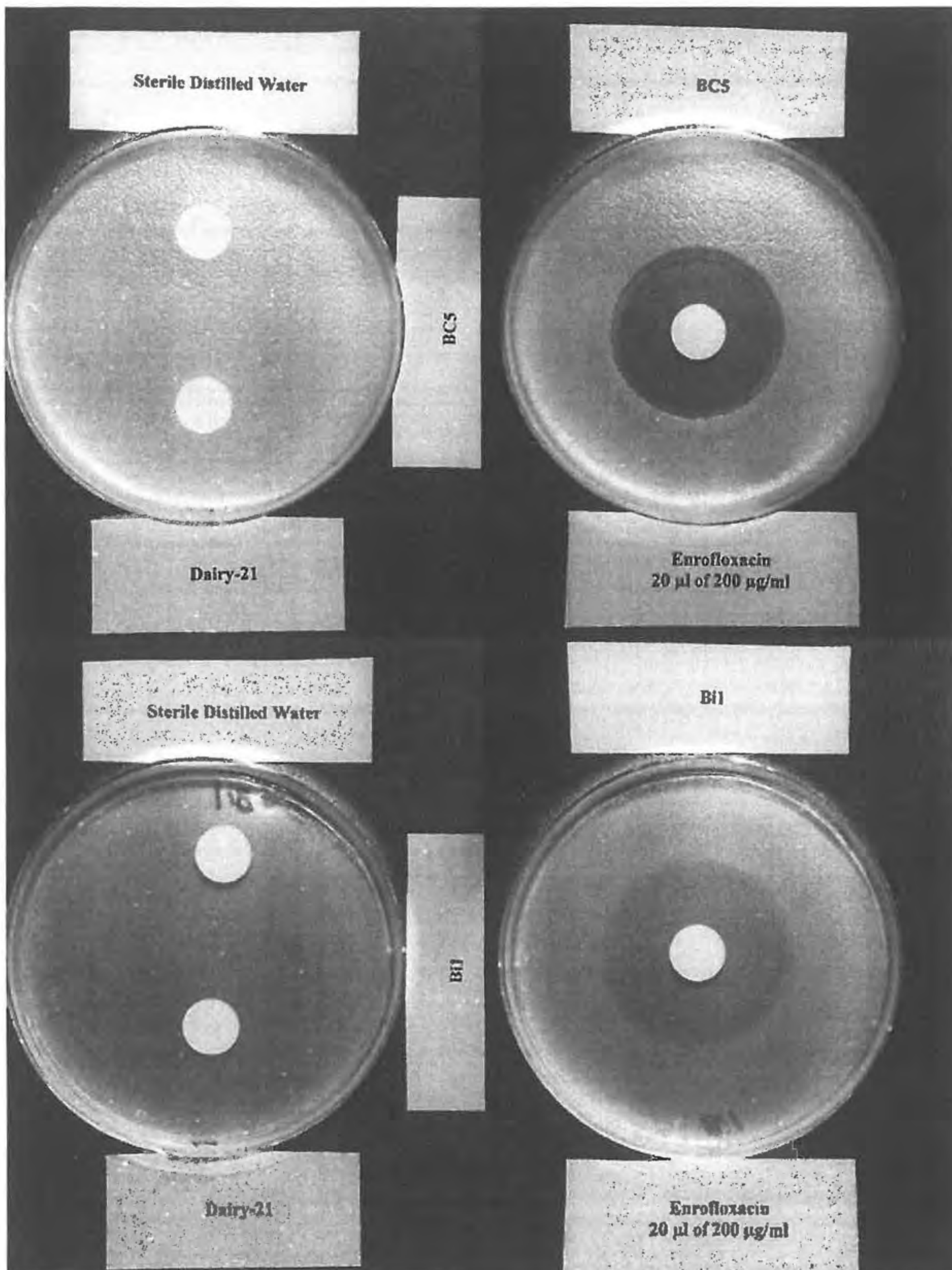
APPENDIX B: Photos



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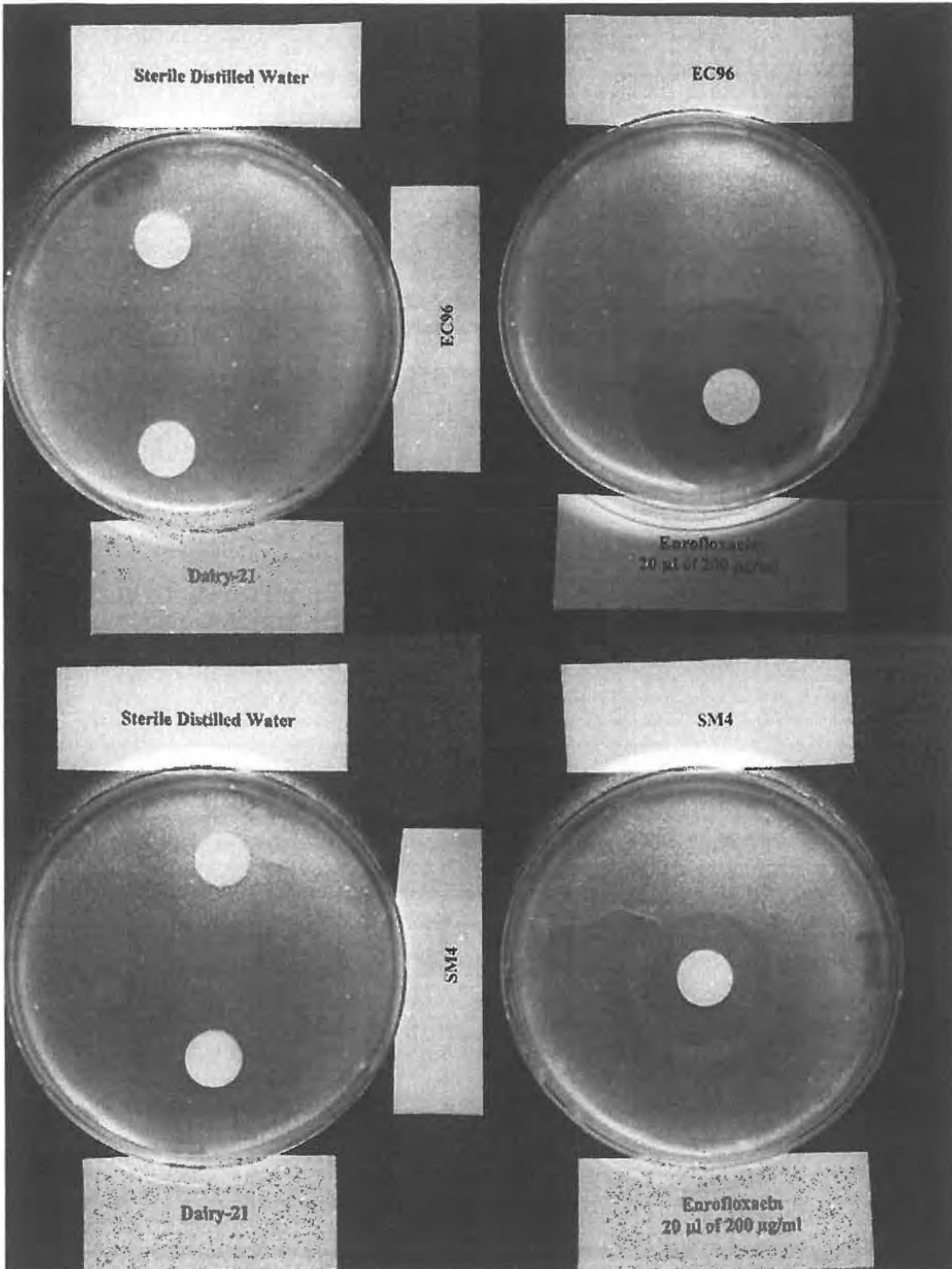

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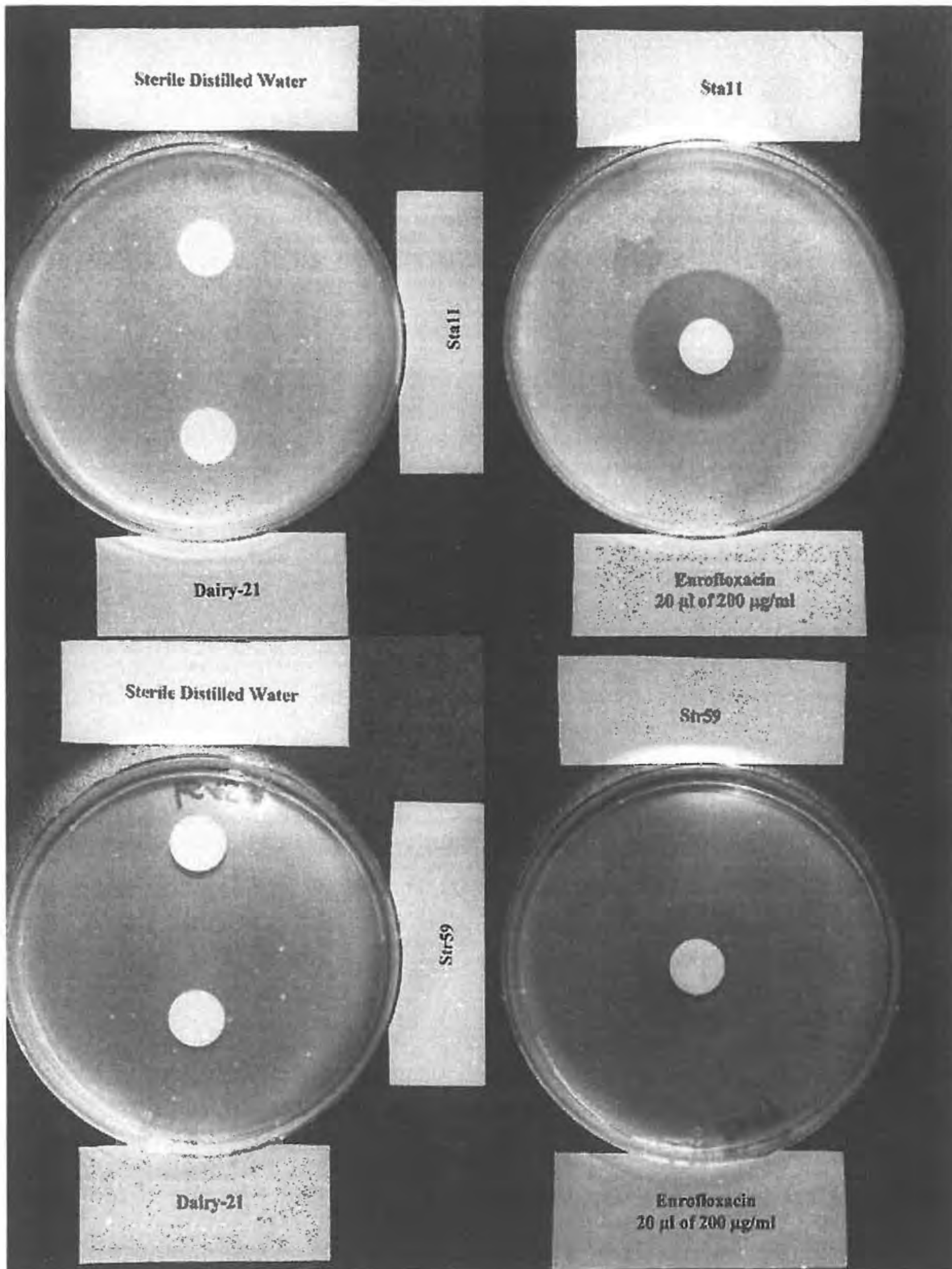

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Appendix 006: Virulence tables from Comparison Organisms to Victors, DFVF, and Phi-Base

Table 1A: Significant Alignments to the Victors Virulence Database to C. krusei / P. kudriavzevii (GCA_003054445)

Query	Subject	%Identity	evalue	Query coverage %
AWU74073.1	gi|68491579|ref|XP_710419.1| potential type 2A-related protein phosphatase [Candida albicans SC5314]	82.54	0	100
AWU77638.1	gi|68480453|ref|XP_715825.1| Ras family GTP-binding protein Rho1p [Candida albicans SC5314]	81.54	4.00E-111	99
AWU76878.1	gi|10383772|ref|NP_009911.2| Leu2p [Saccharomyces cerevisiae S288c]	80.28	0	99
AWU74623.1	gi|68469771|ref|XP_721137.1| likely protein kinase [Candida albicans SC5314]	80.17	0	90

Table 1B: Significant Alignments to the Victors Virulence Database to *C. krusei* / *P. kudriavzevii* (GCA_002166775)

Query	Subject	%Identity	evalue	Query coverage %
OUT22604.1	gi|68480453|ref|XP_715825.1|Ras family GTP-binding protein Rho1p [Candida albicans SC5314]	83.33	2.00E-109	87
OUT23549.1	gi|68491579|ref|XP_710419.1| potential type 2A-related protein phosphatase [Candida albicans SC5314]	82.54	0	100
OUT24422.1	gi|10383772|ref|NP_009911.2| Leu2p [Saccharomyces cerevisiae S288c]	80.28	0	99
OUT20115.1	gi|68469771|ref|XP_721137.1| likely protein kinase [Candida albicans SC5314]	80.17	0	90

Table 1C: Significant Alignments to the Victors Virulence Database to *P. membranifaciens* (GCA_001661235)

Query	Subject	%Identity	evalue	Query coverage %
ODQ45107.1	gi|70984747|ref|XP_747880.1| GATA transcriptional activator AreA [Aspergillus fumigatus Af293]	90.57	5.00E-28	79
ODQ45107.1	gi|799314302|ref|XP_012046597.1| GATA type zinc finger protein asd-4 [Cryptococcus neoformans var. grubii H99]	84.31	2.00E-24	76
ODQ48099.1	gi|68480453|ref|XP_715825.1| Ras family GTP-binding protein Rho1p [Candida albicans SC5314]	83.06	7.00E-111	92
ODQ48101.1	gi|68480453|ref|XP_715825.1| Ras family GTP-binding protein Rho1p [Candida albicans SC5314]	81.52	2.00E-111	92
ODQ47003.1	gi|68491579|ref|XP_710419.1| potential type 2A-related protein phosphatase [Candida albicans SC5314]	81.27	0	100
ODQ44971.1	gi|68469771|ref|XP_721137.1|likely protein kinase [Candida albicans SC5314]	80.74	0	80

Table 2A: **Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *C. krusei / P. kudriavzevii* (GCA_003054445)**

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
AWU78400.1	Q4U2W2_ISSOR|Issatchenkia orientalis|Unknown|Glucan synthase|infection	100	0	100
AWU78204.1	Q5ADS0_CANAL|Candida albicans|Ubiquitin ligase| Ubiquitin ligase| invasive candidal disease	100	1.00E-162	99
AWU75580.1	A7ULH9_CANGY|Candida glycerinogenes|Unknown|Pyrophosphatase|Occasional invasive candidal disease	100	0	100
AWU76275.1	D2JLS4_FUSPO|Fusarium poae|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96
AWU76037.1	D2JLR9_FUSCU|Fusarium culmorum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|yellow dead stems	94.66	1.00E-88	96
AWU76037.1	D2JLR8_FUSCE|Fusarium cerealis|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3| Seedling blight, brown foot rot, and ear blight in wheat and other cereals	94.66	1.00E-88	96
AWU76037.1	D2JLR7_9HYPO|Fusarium camptoceras|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|fescue foot	94.66	1.00E-88	96
AWU76037.1	D2JLR6_FUSBO| Fusarium boothii|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96

Table continued on next page.

Table 2A: **Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by C. krusei / P. kudriavzevii (GCA_003054445) (cont'd)**

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
AWU76037.1	D2JLR4_9HYPO|Fusarium armeniacum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity). |Histone H3|mycotoxins	94.66	1.00E-88	96
AWU76037.1	D2JLR3_9HYPO|Fusarium sp|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96
AWU76275.1	D2JLS9_9HYPO|Fusarium venenatum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|allergy	94.66	1.00E-88	96
AWU76275.1	D2JLS8_9HYPO|Fusarium torulosum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|dry rot	94.66	1.00E-88	96
AWU76275.1	D2JLS7_FUSSP|Fusarium sporotrichioides|he nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|trichothecene	94.66	1.00E-88	96
AWU76275.1	D2JLS6_9HYPO|Fusarium sp|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3| mycotoxins	94.66	1.00E-88	96

Table continued on next page.

Table 2A: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by C. krusei / P. kudriavzevii (GCA_003054445) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
AWU76275.1	D2JLS5_GIBPU|Gibberella pulicaris|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|Tree canker, rot of potatoes	94.66	1.00E-88	96
AWU76275.1	D2JLS3_9HYPO|Fusarium incarnatum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|Anthracnose	94.66	1.00E-88	96
AWU76037.1	D2JLS1_9HYPO|Fusarium kyushuense|he nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96
AWU76275.1	D2JLS2_9HYPO |Fusarium longipes| The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity). |Histone H3|seedling collar rot	94.66	1.00E-88	96
AWU76275.1	D2JLS0_FUSEQ|Fusarium equiseti|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|Rots	94.66	1.00E-88	96
AWU76275.1	D2JLR5_9HYPO |Fusarium avenaceum| The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3 | Blight, head blight of wheat, rots of fruits, stems, and roots, etc	94.66	1.00E-88	96

Table continued on next page.

Table 2A: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by C. krusei / P. kudriavzevii (GCA_003054445) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description|Pfam description|disease)	%identity	evalue	Query coverage %
AWU74027.1	Q59P43_CANAL|Candida albicans|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein |invasive candidal disease	91.51	1.00E-147	99
AWU74027.1	C4YIU6_CANAW|Candida albicans|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle (By similarity).|GTP-binding nuclear protein GSP1/Ran|invasive candidal disease	91.51	1.00E-147	99
AWU74027.1	A7A1H6_YEAS7|Saccharomyces cerevisiae|TP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein |occasional infection	90.95	1.00E-138	98
AWU78336.1	1433_CANAL|Candida albicans| 14-3-3 protein| 14-3-3 protein homolog|invasive candidal disease	87.03	2.00E-155	94
AWU78070.1	Q5A415_CANAL|Candida albicans|Actin|Actin|invasive candidal disease	86.98	0	93
AWU76902.1	F2QXJ4_PICP7|Pichia pastoris|CATALYTIC ACTIVITY: ATP + D-ribose 5-phosphate = AMP + 5-phospho- alpha-D-ribose 1-diphosphate.Belongs to the ribose-phosphate pyrophosphokinase family.|5-phospho-ribosyl-1(Alpha)-pyrophosphate synthetase|occasional infection	86.79	0	99
AWU75937.1	C1GM22_PARBD| Paracoccidioides brasiliensis|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain |Paracoccidioidomycosis	86.36	5.00E-07	85
AWU74027.1	F2QT01_PICP7|Pichia pastoris|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein|occasional infection	86.12	3.00E-135	97

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Table 2A: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by C. krusei / P. kudriavzevii (GCA_003054445) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
AWU77777.1	RL3_YEAST|Saccharomyces cerevisiae|Component of the large ribosomal subunit. Mature ribosomes consist of a small (40S) and a large (60S) subunit. The 40S subunit contains 32 different proteins (encoded by 56 genes) and 1 molecule of RNA (18S). The 60S subunit contains 46 different proteins (encoded by 81 genes) and 3 molecules of RNA (25S, 5.8S and 5S).|60S ribosomal protein L3 |occasional infection	85.27	0	99
AWU74027.1	C5GQ05_AJEDR|Ajellomyces dermatitidis|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein |cutaneous Blastomyces dermatitidis infection	85.05	4.00E-134	99
AWU76902.1	Q5ALK3_CANAL|Candida albicans|CATALYTIC ACTIVITY: ATP + D-ribose 5-phosphate = AMP + 5-phospho- alpha-D-ribose 1-diphosphate.Belongs to the ribose-phosphate pyrophosphokinase family.|Ribose phosphate diphosphokinase subunit|invasive candidal disease	84.95	0	99
AWU76902.1	C4YJK4_CANAW|Candida albicans|CATALYTIC ACTIVITY: ATP + D-ribose 5-phosphate = AMP + 5-phospho- alpha-D-ribose 1-diphosphate.Belongs to the ribose-phosphate pyrophosphokinase family. Ribose phosphate diphosphokinase subunit|invasive candidal disease	84.95	0	99
AWU76902.1	A3LTI2_PICST|Scheffersomyces stipitis|CATALYTIC ACTIVITY: ATP + D-ribose 5-phosphate = AMP + 5-phospho- alpha-D-ribose 1-diphosphate.Belongs to the ribose-phosphate pyrophosphokinase family. Ribose phosphate diphosphokinase subunit|occasional infection	84.95	0	99
AWU76331.1	Q8J031_PICAN|Pichia angusta|GTP-binding protein|GTP-binding protein|occasional infection	84.8	5.00E-129	100

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Table 2A: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by C. krusei / P. kudriavzevii (GCA_003054445) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
AWU74027.1	C0SA80_PARBP|Paracoccidioides brasiliensis|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein|Paracoccidioidomycosis	83.72	3.00E-127	100
AWU74027.1	C1GCT8_PARBD|Paracoccidioides brasiliensis|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein|Paracoccidioidomycosis	83.72	3.00E-127	100
AWU78253.1	F2QRC5_PICP7|Pichia pastoris|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase|occasional infection	82.76	0	95
AWU74073.1	Q59KY8_CANAL|Candida albicans|CATALYTIC ACTIVITY: A phosphoprotein + H(2)O = a protein + phosphate.Belongs to the PPP phosphatase family.| Serine/threonine-protein phosphatase SIT4 EC=3.1.3.16 |invasive candidal disease	82.54	0	100
AWU75397.1	HSP90_CANAL|Candida albicans|Molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction. Undergoes a functional cycle that is linked to its ATPase activity. This cycle probably induces conformational changes in the client proteins, thereby causing their activation. Interacts dynamically with various co-chaperones that modulate its substrate recognition, ATPase cycle and chaperone function (By similarity).|Heat shock protein 90 homolog |invasive candidal disease	82.53	0	99
AWU75937.1	A3LTP2_PICST|Scheffersomyces stipitis |Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain|occasional infection	82.31	0	99

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Table 2A: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by C. krusei / P. kudriavzevii (GCA_003054445) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
AWU78253.1	C5NSJ4_PICPA|Pichia pastoris|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase |occasional infection	82.1	0	96
AWU75937.1	A6ZLY0_YEAS7|Saccharomyces cerevisiae|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain| occasional infection	81.86	0	99
AWU74623.1	HOG1_CRYPA|Cryphonectria parasitica|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression. CATALYTIC ACTIVITY: ATP + a protein = ADP + a phosphoprotein.|Mitogen-activated protein kinase HOG1|Chestnut blight	81.79	0	89
AWU74623.1	HOG1_BOTFB|Botryotinia fuckeliana|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression. CATALYTIC ACTIVITY: ATP + a protein = ADP + a phosphoprotein.|Mitogen-activated protein kinase HOG1|Grey mould	81.52	0	88

Table continued on next page.

Table 2A: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by C. krusei / P. kudriavzevii (GCA_003054445) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
AWU75937.1	B9WMS9_CANDC|Candida dubliniensis|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain|leptomeningeal disease,occasional invasive candidal disease	81.41	0	99
AWU78253.1	A6ZYV3_YEAS7|Saccharomyces cerevisiae|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase|occasional infection	81.19	0	95
AWU78253.1	C7GMI1_YEAS2|Saccharomyces cerevisiae|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase|occasional infection	80.88	0	95
AWU75937.1	Q5A401_CANAL|Candida albicans|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain|invasive candidal disease	80.38	0	71
AWU75937.1	C4YMU9_CANAW|Candida albicans|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain|invasive candidal disease	80.38	0	71
AWU75580.1	C4YKG1_CANAW|Candida albicans|Pyrophosphatase|Inorganic pyrophosphatase | invasive candidal disease	80.35	3.00E-167	99

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Table 2A: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by C. krusei / P. kudriavzevii (GCA_003054445) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
AWU74623.1	HOG1_CANAL|Candida albicans|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression. CATALYTIC ACTIVITY: ATP + a protein = ADP + a phosphoprotein.|Mitogen-activated protein kinase HOG1|invasive candidal disease	80.17	0	90

Table 2B: **Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *C. krusei / P. kudriavzevii* (GCA_002166775)**

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
OUT21010.1	A7ULH9_CANGY|Candida glycerinogenes|Unknown|Pyrophosphatase|Occasional invasive candidal disease	100	0	100
OUT21999.1	Q5ADS0_CANAL |Candida albicans|Ubiquitin ligase| Ubiquitin ligase| invasive candidal disease	99.56	1.00E-157	99
OUT21806.1	Q4U2W2_ISSOR|Issatchenkia orientalis|Unknown|Glucan synthase|infection	98.78	0	100
OUT23853.1	D2JLR4_9HYPO|Fusarium armeniacum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity). |Histone H3|mycotoxins	94.66	1.00E-88	96
OUT23853.1	D2JLS2_9HYPO |Fusarium longipes| The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity). |Histone H3|seedling collar rot	94.66	1.00E-88	96
OUT23853.1	D2JLR5_9HYPO |Fusarium avenaceum| The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3 | Blight, head blight of wheat, rots of fruits, stems, and roots, etc	94.66	1.00E-88	96
OUT23853.1	D2JLR6_FUSBO| Fusarium boothii|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96

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Table 2B: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *C. krusei / P. kudriavzevii* (GCA_002166775) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
OUT23853.1	D2JLR7_9HYPO|Fusarium camptoceras|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|fescue foot	94.66	1.00E-88	96
OUT23853.1	D2JLR8_FUSCE|Fusarium cerealis|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3| Seedling blight, brown foot rot, and ear blight in wheat and other cereals	94.66	1.00E-88	96
OUT23853.1	D2JLR9_FUSCU |Fusarium culmorum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|yellow dead stems	94.66	1.00E-88	96
OUT23853.1	D2JLS0_FUSEQ|Fusarium equiseti|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|Rots	94.66	1.00E-88	96
OUT23853.1	D2JLS1_9HYPO|Fusarium kyushuense|he nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96
OUT23853.1	D2JLS9_9HYPO|Fusarium venenatum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|allergy	94.66	1.00E-88	96

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Table 2B: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *C. krusei / P. kudriavzevii* (GCA_002166775) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
OUT23853.1	D2JLS8_9HYPO|Fusarium torulosum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|dry rot	94.66	1.00E-88	96
OUT23853.1	D2JLS7_FUSSP|Fusarium sporotrichioides|he nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|trichothecene	94.66	1.00E-88	96
OUT23853.1	D2JLS6_9HYPO|Fusarium sp|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3| mycotoxins	94.66	1.00E-88	96
OUT23853.1	D2JLS5_GIBPU|Gibberella pulicaris|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|Tree canker, rot of potatoes	94.66	1.00E-88	96
OUT23853.1	D2JLS4_FUSPO|Fusarium poae|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96
OUT23853.1	D2JLR3_9HYPO|Fusarium sp|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96

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Table 2B: **Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *C. krusei / P. kudriavzevii* (GCA_002166775) (cont'd)**

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
OUT23853.1	D2JLS3_9HYPO|Fusarium incarnatum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|Anthracnose	94.66	1.00E-88	96
OUT23504.1	C4YIU6_CANAW|Candida albicans|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle (By similarity).|GTP-binding nuclear protein GSP1/Ran|invasive candidal disease	91.51	1.00E-147	99
OUT23504.1	Q59P43_CANAL|Candida albicans|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein |invasive candidal disease	91.51	1.00E-147	99
OUT23504.1	A7A1H6_YEAS7|Saccharomyces cerevisiae|TP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein |occasional infection	90.95	1.00E-138	98
OUT21865.1	1433_CANAL|Candida albicans| 14-3-3 protein| 14-3-3 protein homolog|invasive candidal disease	87.03	2.00E-155	94
OUT22136.1	Q5A415_CANAL|Candida albicans|Actin|Actin|invasive candidal disease	86.98	0	93
OUT24443.1	F2QXJ4_PICP7|Pichia pastoris|CATALYTIC ACTIVITY: ATP + D-ribose 5-phosphate = AMP + 5-phospho- alpha-D-ribose 1-diphosphate.Belongs to the ribose-phosphate pyrophosphokinase family.|5-phospho-ribosyl-1(Alpha)-pyrophosphate synthetase|occasional infection	86.79	0	99

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Table 2B: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *C. krusei / P. kudriavzevii* (GCA_002166775) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
OUT23504.1	F2QT01_PICP7|Pichia pastoris|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein|occasional infection	86.12	3.00E-135	97
OUT22741.1	RL3_YEAST|Saccharomyces cerevisiae|Component of the large ribosomal subunit. Mature ribosomes consist of a small (40S) and a large (60S) subunit. The 40S subunit contains 32 different proteins (encoded by 56 genes) and 1 molecule of RNA (18S). The 60S subunit contains 46 different proteins (encoded by 81 genes) and 3 molecules of RNA (25S, 5.8S and 5S).|60S ribosomal protein L3 |occasional infection	85.27	0	99
OUT23504.1	C5GQ05_AJEDR|Ajellomyces dermatitidis|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein |cutaneous Blastomyces dermatitidis infection	85.05	4.00E-134	99
OUT24443.1	Q5ALK3_CANAL|Candida albicans|CATALYTIC ACTIVITY: ATP + D-ribose 5-phosphate = AMP + 5-phospho- alpha-D-ribose 1-diphosphate.Belongs to the ribose-phosphate pyrophosphokinase family.|Ribose phosphate diphosphokinase subunit|invasive candidal disease	84.95	0	99
OUT24443.1	C4YJK4_CANAW|Candida albicans|CATALYTIC ACTIVITY: ATP + D-ribose 5-phosphate = AMP + 5-phospho- alpha-D-ribose 1-diphosphate.Belongs to the ribose-phosphate pyrophosphokinase family. Ribose phosphate diphosphokinase subunit|invasive candidal disease	84.95	0	99
OUT24443.1	A3LTI2_PICST|Scheffersomyces stipitis|CATALYTIC ACTIVITY: ATP + D-ribose 5-phosphate = AMP + 5-phospho- alpha-D-ribose 1-diphosphate.Belongs to the ribose-phosphate pyrophosphokinase family. Ribose phosphate diphosphokinase subunit|occasional infection	84.95	0	99

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Table 2B: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *C. krusei / P. kudriavzevii* (GCA_002166775) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description|Pfam description|disease)	%identity	evalue	Query coverage %
OUT23504.1	C0SA80_PARBP|Paracoccidioides brasiliensis|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein|Paracoccidioidomycosis	83.72	3.00E-127	100
OUT23504.1	C1GCT8_PARBD|Paracoccidioides brasiliensis|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein|Paracoccidioidomycosis	83.72	3.00E-127	100
OUT21948.1	F2QRC5_PICP7|Pichia pastoris|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase|occasional infection	82.76	0	95
OUT23549.1	Q59KY8_CANAL|Candida albicans|CATALYTIC ACTIVITY: A phosphoprotein + H(2)O = a protein + phosphate.Belongs to the PPP phosphatase family.|Serine/threonine-protein phosphatase SIT4 EC=3.1.3.16 |invasive candidal disease	82.54	0	100
OUT21617.1	HSP90_CANAL|Candida albicans|Molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction. Undergoes a functional cycle that is linked to its ATPase activity. This cycle probably induces conformational changes in the client proteins, thereby causing their activation. Interacts dynamically with various co-chaperones that modulate its substrate recognition, ATPase cycle and chaperone function (By similarity).|Heat shock protein 90 homolog |invasive candidal disease	82.53	0	99
OUT21948.1	C5NSJ4_PICPA|Pichia pastoris|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase |occasional infection	82.1	0	96

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Table 2B: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *C. krusei / P. kudriavzevii* (GCA_002166775) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description|Pfam description|disease)	%identity	evalue	Query coverage %
OUT20115.1	HOG1_CRYPA|Cryphonectria parasitica|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression. CATALYTIC ACTIVITY: ATP + a protein = ADP + a phosphoprotein.|Mitogen-activated protein kinase HOG1|Chestnut blight	81.79	0	89
OUT20115.1	HOG1_BOTFB|Botryotinia fuckeliana|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression. CATALYTIC ACTIVITY: ATP + a protein = ADP + a phosphoprotein.|Mitogen-activated protein kinase HOG1|Grey mould	81.52	0	88
OUT21948.1	A6ZYV3_YEAS7|Saccharomyces cerevisiae|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase|occasional infection	81.19	0	95
OUT21948.1	C7GMI1_YEAS2|Saccharomyces cerevisiae|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase|occasional infection	80.88	0	95

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Table 2B: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *C. krusei / P. kudriavzevii* (GCA_002166775) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
OUT21010.1	C4YKG1_CANAW|Candida albicans|Pyrophosphatase|Inorganic pyrophosphatase | invasive candidal disease	80.35	3.00E-167	99
OUT20115.1	HOG1_CANAL|Candida albicans|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression. CATALYTIC ACTIVITY: ATP + a protein = ADP + a phosphoprotein.|Mitogen-activated protein kinase HOG1|invasive candidal disease	80.17	0	90

Table 2C: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *P. membranifaciens* (GCA_001661235)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
ODQ48577.1	Q5ADS0_CANAL|Candida albicans|Ubiquitin ligase| Ubiquitin ligase| invasive candidal disease	98.68	1.00E-47	99
ODQ45107.1	Q7Z9Z4_CANAL|Candida albicans|Nitrogen regulatory GATA-factor|Nitrogen regulatory GATA-factor|invasive candidal disease	96	5.00E-30	75
ODQ45107.1	Q5A432_CANAL|Candida albicans|Transcriptional regulator of nitrogen utilization required for nitrogen catabolite repression and utilization of isoleucine, tyrosine and tryptophan as nitrogen sources. Controls expression of the MEP2 ammonium permease, the DUR1,2 urea amidolyase, and the transcription factor STP1, which in turn mediates SAP2 expression, a long-known virulence attribute of C.albicans. Influences the filamentation process depending upon the nitrogen sources available. Required for virulence in a mouse systemic infection model| Transcriptional regulatory protein GAT1|invasive candidal disease	96	3.00E-30	75
ODQ44720.1	D2JLS1_9HYPO|Fusarium kyushuense|he nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96
ODQ44720.1	D2JLS8_9HYPO|Fusarium torulosum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|dry rot	94.66	1.00E-88	96
ODQ44720.1	D2JLS7_FUSSP|Fusarium sporotrichioides|he nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|trichothecene	94.66	1.00E-88	96

Table continued on next page.

Table 2C: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *P. membranifaciens* (GCA_001661235) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
ODQ44720.1	D2JLS5_GIBPU|Gibberella pulicaris|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|Tree canker, rot of potatoes	94.66	1.00E-88	96
ODQ44720.1	D2JLS4_FUSPO|Fusarium poae|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96
ODQ44720.1	D2JLS3_9HYPO|Fusarium incarnatum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|Anthracnose	94.66	1.00E-88	96
ODQ44720.1	D2JLS2_9HYPO |Fusarium longipes| The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity). |Histone H3|seedling collar rot	94.66	1.00E-88	96
ODQ44720.1	D2JLR8_FUSCE|Fusarium cerealis|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3| Seedling blight, brown foot rot, and ear blight in wheat and other cereals	94.66	1.00E-88	96
ODQ44720.1	D2JLR9_FUSCU|Fusarium culmorum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|yellow dead stems	94.66	1.00E-88	96

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Table 2C: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *P. membranifaciens* (GCA_001661235) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
ODQ44498.1	D2JLR3_9HYPO|Fusarium sp|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96
ODQ44720.1	D2JLR7_9HYPO|Fusarium camptoceras|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|fescue foot	94.66	1.00E-88	96
ODQ44720.1	D2JLR6_FUSBO| Fusarium boothii|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|mycotoxins	94.66	1.00E-88	96
ODQ44720.1	D2JLR5_9HYPO |Fusarium avenaceum| The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3 | Blight, head blight of wheat, rots of fruits, stems, and roots, etc	94.66	1.00E-88	96
ODQ44720.1	D2JLR4_9HYPO|Fusarium armeniacum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity). |Histone H3|mycotoxins	94.66	1.00E-88	96
ODQ44720.1	D2JLS9_9HYPO|Fusarium venenatum|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|allergy	94.66	1.00E-88	96

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Table 2C: **Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *P. membranifaciens* (GCA_001661235) (cont'd)**

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
ODQ44720.1	D2JLS0_FUSEQ|Fusarium equiseti|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3|Rots	94.66	1.00E-88	96
ODQ44498.1	D2JLS6_9HYPO|Fusarium sp|The nucleosome is a histone octamer containing two molecules each of H2A, H2B, H3 and H4 assembled in one H3-H4 heterotetramer and two H2A-H2B heterodimers. The octamer wraps approximately 147 bp of DNA (By similarity).|Histone H3| mycotoxins	94.66	1.00E-88	96
ODQ44781.1	A7XLC8_PICAN|Pichia angusta|Putative Mig1p zinc binding protein|Putative Mig1p|occasional infection	92.73	1.00E-34	100
ODQ48734.1	C4YIU6_CANAW|Candida albicans|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle (By similarity).|GTP-binding nuclear protein GSP1/Ran|invasive candidal disease	91.51	2.00E-148	99
ODQ48734.1	Q59P43_CANAL|Candida albicans|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein |invasive candidal disease	91.51	2.00E-148	99
ODQ47685.1	Q8J031_PICAN|Pichia angusta|GTP-binding protein|GTP-binding protein|occasional infection	90.15	9.00E-138	100
ODQ47460.1	A7ULH9_CANGY|Candida glycerinogenes|Unknown|Pyrophosphatase|Occasional invasive candidal disease	89.82	0	100

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Table 2C: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *P. membranifaciens* (GCA_001661235) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
ODQ48734.1	A7A1H6_YEAS7|Saccharomyces cerevisiae|TP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein |occasional infection	89.72	2.00E-139	99
ODQ44781.1	C4YKV7_CANAW|Candida albicans|Uncharacterized protein|Uncharacterized protein|invasive candidal disease	89.09	3.00E-31	100
ODQ46905.1	Q4U2W2_ISSOR|Issatchenkia orientalis|Unknown|Glucan synthase|infection	88.38	0	95
ODQ48383.1	B9WHL1_CANDC|Candida dubliniensis| Mig1 transcriptional repressor|unknown|leptomeningeal disease,occasional invasive candidal disease	87.27	2.00E-28	100
ODQ48383.1	Q5AG61_CANAL|Candida albicans|N/A deleted entry|N/A deleted entry|invasive candidal disease	87.27	2.00E-28	100
ODQ48383.1	A3LVN8_PICST|Scheffersomyces stipitis|nucleic acid binding|Transcription factor involved in glucose repression|occasional infection	87.27	5.00E-29	100
ODQ48383.1	F2QPW6_PICP7|Pichia pastoris|nucleic acid binding| Transcription factor| occasional infection	87.27	1.00E-30	100
ODQ44781.1	Q96TV0_SCHOC|Schwanniomyces occidentalis|nucleic acid binding|Mig1 protein|occasional infection	87.27	1.00E-31	100
ODQ45787.1	Q5A415_CANALQ5A415_CANAL|Candida albicans|Actin|Actin|invasive candidal disease	86.7	0	93
ODQ48734.1	F2QT01_PICP7F2QT01_PICP7|Pichia pastoris|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein|occasional infection	86.12	1.00E-135	97

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Table 2C: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *P. membranifaciens* (GCA_001661235) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
ODQ48734.1	C5GQ05_AJEDR|Ajellomyces dermatitidis|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein |cutaneous Blastomyces dermatitidis infection	85.51	1.00E-134	99
ODQ44781.1	C1G7T1_PARBD|Paracoccidioides brasiliensis|Uncharacterized protein|N/A no entry|Paracoccidioidomycosis	85.45	2.00E-29	100
ODQ44781.1	C5GNK0_AJEDR|Ajellomyces dermatitidis|nucleic acid binding Zinc-finger protein CreA/MIG| N/A no entry| cutaneous Blastomyces dermatitidis infection	85.45	7.00E-30	100
ODQ44411.1	A3LTP2_PICST|Scheffersomyces stipitis |Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain|occasional infection	83.82	0	99
ODQ48734.1	C1GCT8_PARBD|Paracoccidioides brasiliensis|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein|Paracoccidioidomycosis	83.72	3.00E-127	100
ODQ48734.1	C0SA80_PARBP|Paracoccidioides brasiliensis|GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.|GTP-binding nuclear protein|Paracoccidioidomycosis	83.72	3.00E-127	100
ODQ47280.1	RL3_YEAST|Saccharomyces cerevisiae|Component of the large ribosomal subunit. Mature ribosomes consist of a small (40S) and a large (60S) subunit. The 40S subunit contains 32 different proteins (encoded by 56 genes) and 1 molecule of RNA (18S). The 60S subunit contains 46 different proteins (encoded by 81 genes) and 3 molecules of RNA (25S, 5.8S and 5S).|60S ribosomal protein L3 |occasional infection	83.46	0	99

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Table 2C: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *P. membranifaciens* (GCA_001661235) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description|Pfam description|disease)	%identity	evalue	Query coverage %
ODQ44411.1	B9WMS9_CANDC|Candida dubliniensis|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain|leptomeningeal disease,occasional invasive candidal disease	83.37	0	99
ODQ45107.1	Q8J1X6_COLLN|Colletotrichum lindemuthianum| Major nitrogen regulatory protein|Major nitrogen regulatory protein|Leaf, stem and pod anthracnose	83.08	1.00E-30	97
ODQ44411.1	A6ZLY0_YEAS7|Saccharomyces cerevisiae|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain| occasional infection	82.7	0	99
ODQ46960.1	1433_CANAL|Candida albicans| 14-3-3 protein| 14-3-3 protein homolog|invasive candidal disease	82.69	1.00E-156	100
ODQ44411.1	Q5A401_CANAL|Candida albicans|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain|invasive candidal disease	82.5	0	72
ODQ44411.1	C4YMU9_CANAW|Candida albicans|Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain (By similarity).|Tubulin alpha chain|invasive candidal disease	82.5	0	72

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Table 2C: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *P. membranifaciens* (GCA_001661235) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
ODQ45444.1	HSP90_CANAL|Candida albicans|Molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction. Undergoes a functional cycle that is linked to its ATPase activity. This cycle probably induces conformational changes in the client proteins, thereby causing their activation. Interacts dynamically with various co-chaperones that modulate its substrate recognition, ATPase cycle and chaperone function (By similarity).|Heat shock protein 90 homolog |invasive candidal disease	81.92	0	99
ODQ44971.1	HOG1_BOTFB|Botryotinia fuckeliana|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression. CATALYTIC ACTIVITY: ATP + a protein = ADP + a phosphoprotein.|Mitogen-activated protein kinase HOG1|Grey mould	81.52	0	78
ODQ48630.1	C7GMI1_YEAS2|Saccharomyces cerevisiae|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase|occasional infection	81.5	0	80
ODQ48630.1	A6ZYV3_YEAS7|Saccharomyces cerevisiae|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase|occasional infection	81.5	0	80

Table continued on next page.

Table 2C: Non Redundant Subject Sequence Hits to the Database of Fungal Virulence Factors by *P. membranifaciens* (GCA_001661235) (cont'd)

Query	Subject (UniprotID|Organism|Uniprot description| Pfam description|disease)	%identity	evalue	Query coverage %
ODQ44971.1	HOG1_CRYPA|Cryphonectria parasitica|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression. CATALYTIC ACTIVITY: ATP + a protein = ADP + a phosphoprotein.|Mitogen-activated protein kinase HOG1|Chestnut blight	81.46	0	80
ODQ47003.1	Q59KY8_CANAL|Candida albicans|CATALYTIC ACTIVITY: A phosphoprotein + H(2)O = a protein + phosphate.Belongs to the PPP phosphatase family.| Serine/threonine-protein phosphatase SIT4 EC=3.1.3.16 |invasive candidal disease	81.27	0	100
ODQ47685.1	F2QX13_PICP7|Pichia pastoris|Rab family GTPase|Rab family GTPase|occasional infection	80.88	1.00E-116	100
ODQ44971.1	HOG1_CANAL|Candida albicans|Mitogen-activated protein kinase involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment. Controls osmotic regulation of transcription of target genes (By similarity). Involved in the virulence and conidia formation. Mediates tannic acid-induced laccase expression and cryparin expression. CATALYTIC ACTIVITY: ATP + a protein = ADP + a phosphoprotein.|Mitogen-activated protein kinase HOG1|invasive candidal disease	80.74	0	80
ODQ47460.1	C4YKG1_CANAW|Candida albicans|Pyrophosphatase|Inorganic pyrophosphatase | invasive candidal disease	80.35	7.00E-167	99
ODQ48630.1	F2QRC5_PICP7|Pichia pastoris|CATALYTIC ACTIVITY: Thioredoxin + NADP(+) = thioredoxin disulfide + NADPH.Belongs to the class-II pyridine nucleotide-disulfide oxidoreductase family.|Thioredoxin reductase|occasional infection	80.25	0	80

Table 3A: Hits to the Pathogen-Host Interactions Database by *C. krusei / P. kudriavzevii* (GCA_003054445)

Query	Subject|Gene ID|PhiBase ID| Gene|Pathogen ID|Species|Phenotype	%identity	evalue	Query coverage %
AWU77466.1	Q4WJS6|PHI:6753|GlcA|746128|Aspergillus_fumigatus|lethal	94.37	0	96
AWU77832.1	Q5AND9|PHI:7009|arf2|5476|Candida_albicans|reduced_virulence	89.5	4.00E-120	100
AWU75563.1	Q5AJB1|PHI:3321|Tfp1|5476|Candida_albicans|loss_of_pathogenicity	87.52	0	100
AWU78336.1	O42766|PHI:3497__PHI:2816|Bmh1p__BMH1|5476|Candida_albicans | reduced_virulence__lethal	87.03	5.00E-155	94
AWU75151.1	I1S1V9|PHI:1604|GzOB045|5518|Fusarium_graminearum| unaffected_pathogenicity	86.21	2.00E-89	100
AWU73775.1	Q4HTT1|PHI:1468|GzCCAAT008|5518|Fusarium_graminearum| unaffected_pathogenicity	85.59	7.00E-69	89
AWU74973.1	Q4HTT1|PHI:1468|GzCCAAT008|5518|Fusarium_graminearum| unaffected_pathogenicity	85.59	7.00E-69	89
AWU75591.1	Q5A5Q8|PHI:6076|RPS41|5476|Candida_albicans| reduced_virulence	82.82	6.00E-167	100
AWU74623.1	E9EYM7|PHI:5427|Mero-Hog1|568076|Metarhizium_robertsii| reduced_virulence	82.56	0	89
AWU74073.1	Q59KY8|PHI:378|SIT4|5476|Candida_albicans| reduced_virulence	82.54	0	100
AWU74623.1	Q6FIU2|PHI:4625__PHI:8395|CgHog1__Cghog1|5478| Candida_glabrata|reduced_virulence__unaffected_pathogenicity	82.27	0	91
AWU74623.1	Q4W6D3|PHI:2365|Hog1|5016|Bipolaris_maydis| reduced_virulence	82.27	0	89
AWU75671.1	Q5AMP3|PHI:7011|arl1|5476|Candida_albicans| reduced_virulence	82.16	1.00E-111	100
AWU74623.1	Q56R42|PHI:497|HOG1|5207|Cryptococcus_neoformans| reduced_virulence	82.11	0	88
AWU74623.1	M2SJ60|PHI:4779|Cshog1|45130|Bipolaris_sorokiniana| reduced_virulence__unaffected_pathogenicity	81.98	0	89
AWU74623.1	Q875L0|PHI:342|CpMK1|5116|Cryphonectria_parasitica| reduced_virulence	81.79	0	89

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Table 3A: Hits to the Pathogen-Host Interactions Database by *C. krusei / P. kudriavzevii* (GCA_003054445) (cont'd)

Query	Subject|Gene ID|PhiBase ID| Gene|Pathogen ID|Species|Phenotype	%identity	evalue	Query coverage %
AWU74623.1	P0C431|PHI:1005__PHI:2327|FgHOG1__HOG1|5518| Fusarium_graminearum|reduced_virulence__unaffected_pathogenicit y	81.79	0	89
AWU77638.1	O42825|PHI:270|RHO1|5476|Candida_albicans| loss_of_pathogenicity	81.54	6.00E-111	99
AWU74623.1	A1IVT7|PHI:1031|bcSAK1|40559|Botrytis_cinerea| reduced_virulence	81.52	0	88
AWU74623.1	A0A0D2XQS0|PHI:6317|Hog1|5507|Fusarium_oxysporum| reduced_virulence	81.5	0	89
AWU74623.1	Q0U4L8|PHI:7568|HOG1|13684|Parastagonospora_nodorum| unaffected_pathogenicity	81.45	0	89
AWU77899.1	G4NJ14|PHI:2188|MoMCM1|318829|Magnaporthe_oryzae| reduced_virulence__loss_of_pathogenicity	81.37	1.00E-53	35
AWU74623.1	Q6PWX2|PHI:7280|hog1|176275|Beauveria_bassiana| reduced_virulence	81.21	0	89
AWU74623.1	Q1KTF2|PHI:1043|MgHog1|1047171|Zymoseptoria_tritici| loss_of_pathogenicity	81.1	0	89
AWU76878.1	P04173|PHI:504|LEU2|4932|Saccharomyces_cerevisiae| reduced_virulence	80.28	0	99
AWU74623.1	Q92207|PHI:149|HOG1|5476|Candida_albicans| reduced_virulence	80.17	0	90

Table 3B: Hits to the Pathogen-Host Interactions Database by *C. krusei / P. kudriavzevii* (GCA_002166775)

Query	Subject|Gene ID|PhiBase ID| Gene|Pathogen ID|Species|Phenotype	%identity	evalue	Query coverage %
OUT22443.1	Q4WJS6|PHI:6753|GlcA|746128|Aspergillus_fumigatus|lethal	91.72	0	96
OUT22794.1	Q5AND9|PHI:7009|arf2|5476|Candida_albicans|reduced_virulence	89.5	4.00E-120	100
OUT21865.1	O42766|PHI:3497__PHI:2816|Bmh1p__BMH1|5476|Candida_albicans| reduced_virulence__lethal	87.03	5.00E-155	94
OUT21383.1	I1S1V9|PHI:1604|GzOB045|5518|Fusarium_graminearum| unaffected_pathogenicity	86.21	2.00E-89	100
OUT21219.1	Q4HTT1|PHI:1468|GzCCAAT008|5518|Fusarium_graminearum| unaffected_pathogenicity	85.59	7.00E-69	89
OUT23265.1	Q4HTT1|PHI:1468|GzCCAAT008|5518|Fusarium_graminearum| unaffected_pathogenicity	85.59	7.00E-69	89
OUT22604.1	O42825|PHI:270|RHO1|5476|Candida_albicans| loss_of_pathogenicity	83.33	3.00E-109	87
OUT21000.1	Q5A5Q8|PHI:6076|RPS41|5476|Candida_albicans| reduced_virulence	83.2	6.00E-155	100
OUT20115.1	E9EYM7|PHI:5427|Mero-Hog1|568076|Metarhizium_robertsii| reduced_virulence	82.56	0	89
OUT23549.1	Q59KY8|PHI:378|SIT4|5476|Candida_albicans| reduced_virulence	82.54	0	100
OUT20115.1	Q6FIU2|PHI:4625__PHI:8395|CgHog1__Cghog1|5478| Candida_glabrata|reduced_virulence__unaffected_pathogenicity	82.27	0	91
OUT20115.1	Q4W6D3|PHI:2365|Hog1|5016|Bipolaris_maydis| reduced_virulence	82.27	0	89
OUT20115.1	Q56R42|PHI:497|HOG1|5207|Cryptococcus_neoformans| reduced_virulence	82.11	0	88
OUT20115.1	M2SJ60|PHI:4779|Cshog1|45130|Bipolaris_sorokiniana| reduced_virulence__unaffected_pathogenicity	81.98	0	89
OUT20115.1	Q875L0|PHI:342|CpMK1|5116|Cryphonectria_parasitica| reduced_virulence	81.79	0	89
OUT20115.1	P0C431|PHI:1005__PHI:2327|FgHOG1__HOG1|5518| Fusarium_graminearum|reduced_virulence__unaffected_pathogenicity	81.79	0	89
OUT20115.1	A1IVT7|PHI:1031|bcSAK1|40559|Botrytis_cinerea| reduced_virulence	81.52	0	88
OUT20115.1	A0A0D2XQS0|PHI:6317|Hog1|5507|Fusarium_oxysporum| reduced_virulence	81.5	0	89
OUT20115.1	Q0U4L8|PHI:7568|HOG1|13684|Parastagonospora_nodorum| unaffected_pathogenicity	81.45	0	89

Table continued on next page.

Table 3B: Hits to the Pathogen-Host Interactions Database by *C. krusei / P. kudriavzevii* (GCA_002166775) (cont'd)

Query	Subject|Gene ID|PhiBase ID| Gene|Pathogen ID|Species|Phenotype	%identity	evalue	Query coverage %
OUT20115.1	Q6PWX2|PHI:7280|hog1|176275|Beauveria_bassiana| reduced_virulence	81.21	0	89
OUT20115.1	Q1KTF2|PHI:1043|MgHog1|1047171|Zymoseptoria_tritici| loss_of_pathogenicity	81.1	0	89
OUT24422.1	P04173|PHI:504|LEU2|4932|Saccharomyces_cerevisiae| reduced_virulence	80.28	0	99
OUT20115.1	Q92207|PHI:149|HOG1|5476|Candida_albicans| reduced_virulence	80.17	0	90

Table 3C: Hits to the Pathogen-Host Interactions Database by *P. membranifaciens* (GCA_001661235)

Query	Subject|Gene ID|PhiBase ID| Gene|Pathogen ID|Species|Phenotype	%identity	evalue	Query coverage %
ODQ45107.1	Q7Z9Z4|PHI:303|GAT1|5476|Candida_albicans|reduced_virulence	96	2.00E-29	75
ODQ49571.1	Q4WJS6|PHI:6753|GlcA|746128|Aspergillus_fumigatus|lethal	94.65	0	94
ODQ45107.1	Q9HEW7|PHI:5562|Nrf1|5499|Passalora_fulva|unaffected_pathogenicity	94.34	3.00E-29	79
ODQ45107.1	Q01168|PHI:52__PHI:2992|NUT1__MGG_02755.6|318829| Magnaporthe_oryzae|unaffected_pathogenicity__reduced_virulence	94.34	9.00E-29	79
ODQ47222.1	Q5AND9|PHI:7009|arf2|5476|Candida_albicans|reduced_virulence	90.61	9.00E-121	100
ODQ44781.1	I1RZ85|PHI:1412|GzC2H079|5518|Fusarium_graminearum| unaffected_pathogenicity	87.27	1.00E-30	100
ODQ44781.1	J9MZK9|PHI:2455|CRE1|5507|Fusarium_oxysporum| unaffected_pathogenicity	87.27	2.00E-30	100
ODQ44781.1	J4W5V1|PHI:3992__PHI:8630|BbcreA__creA|176275| Beauveria_bassiana|reduced_virulence	87.27	2.00E-30	100
ODQ46514.1	Q4HTT1|PHI:1468|GzCCAAT008|5518|Fusarium_graminearum| unaffected_pathogenicity	86.44	5.00E-69	89
ODQ48994.1	Q4HTT1|PHI:1468|GzCCAAT008|5518|Fusarium_graminearum| unaffected_pathogenicity	86.44	5.00E-69	89
ODQ46692.1	I1S1V9|PHI:1604|GzOB045|5518|Fusarium_graminearum| unaffected_pathogenicity	86.21	3.00E-89	100
ODQ48383.1	I1RZ85|PHI:1412|GzC2H079|5518|Fusarium_graminearum| unaffected_pathogenicity	85.45	2.00E-28	100
ODQ48383.1	J9MZK9|PHI:2455|CRE1|5507|Fusarium_oxysporum| unaffected_pathogenicity	85.45	3.00E-28	100
ODQ48383.1	J4W5V1|PHI:3992__PHI:8630|BbcreA__creA|176275| Beauveria_bassiana|reduced_virulence	85.45	5.00E-28	100
ODQ45107.1	J9VI93|PHI:3515|Gat1|5207|Cryptococcus_neoformans| unaffected_pathogenicity	84.31	2.00E-24	76
ODQ44971.1	Q6FIU2|PHI:4625__PHI:8395|CgHog1__Cghog1|5478| Candida_glabrata|reduced_virulence__unaffected_pathogenicity	84.18	0	80
ODQ44971.1	Q4W6D3|PHI:2365|Hog1|5016|Bipolaris_maydis|reduced_virulence	83.14	0	78
ODQ45107.1	T0KMX6|PHI:6987|CgareA|474922|Colletotrichum_gloeosporioides| reduced_virulence__unaffected_pathogenicity	83.08	2.00E-30	97
ODQ45107.1	Q8J1X6|PHI:287|CLNR1|290576|Colletotrichum_lindemuthianum| loss_of_pathogenicity	83.08	3.00E-30	97

Table continued on next page.

Table 3C: Hits to the Pathogen-Host Interactions Database by P. membranifaciens (GCA_001661235) (cont'd)

Query	Subject|Gene ID|PhiBase ID| Gene|Pathogen ID|Species|Phenotype	%identity	evalue	Query coverage %
ODQ48099.1	O42825|PHI:270|RHO1|5476|Candida_albicans| loss_of_pathogenicity	83.06	1.00E-110	92
ODQ44971.1	M2SJ60|PHI:4779|Cshog1|45130|Bipolaris_sorokiniana| reduced_virulence__unaffected_pathogenicity	82.85	0	78
ODQ44971.1	Q4WSF6|PHI:6084|sakA|746128|Aspergillus_fumigatus| reduced_virulence	82.75	0	78
ODQ46960.1	O42766|PHI:3497__PHI:2816|Bmh1p__BMH1|5476| Candida_albicans|reduced_virulence__lethal	82.69	5.00E-156	100
ODQ44971.1	Q0U4L8|PHI:7568|HOG1|13684|Parastagonospora_nodorum| unaffected_pathogenicity	82.61	0	79
ODQ44971.1	Q9UV51|PHI:153|OSM1|318829|Magnaporthe_oryzae| unaffected_pathogenicity	82.56	0	78
ODQ44971.1	K9FTS6|PHI:8735|Hog1_(PDIG_79560)|36651|Penicillium_digitatum| reduced_virulence__unaffected_pathogenicity	82.46	0	78
ODQ44971.1	P0C431|PHI:1005__PHI:2327|FgHOG1__HOG1|5518| Fusarium_graminearum|reduced_virulence__unaffected_pathogenicity	82.27	0	78
ODQ47368.1	Q5AMP3|PHI:7011|arl1|5476|Candida_albicans| reduced_virulence	82.16	4.00E-112	100
ODQ44971.1	A0A0D2XQS0|PHI:6317|Hog1|5507|Fusarium_oxysporum| reduced_virulence	81.98	0	78
ODQ47449.1	Q5A5Q8|PHI:6076|RPS41|5476|Candida_albicans| reduced_virulence	81.71	3.00E-162	100
ODQ45107.1	I1RWG5|PHI:1448__PHI:2430|GzGATA006__areA|5518| Fusarium_graminearum|unaffected_pathogenicity__reduced_virulence	81.54	6.00E-30	97
ODQ45107.1	J9MJU9|PHI:2283|AreA|5507|Fusarium_oxysporum| reduced_virulence	81.54	7.00E-30	97
ODQ45107.1	A0A0J9UIM0|PHI:7685|AreA|5507|Fusarium_oxysporum| increased_virulence_(hypervirulence)	81.54	9.00E-30	97
ODQ44971.1	A1IVT7|PHI:1031|bcSAK1|40559|Botrytis_cinerea| reduced_virulence	81.52	0	78
ODQ48101.1	O42825|PHI:270|RHO1|5476|Candida_albicans| loss_of_pathogenicity	81.52	2.00E-111	92
ODQ44971.1	Q875L0|PHI:342|CpMK1|5116|Cryphonectria_parasitica| reduced_virulence	81.46	0	80
ODQ44971.1	Q6PWX2|PHI:7280|hog1|176275|Beauveria_bassiana| reduced_virulence	81.43	0	79
ODQ44971.1	Q1KTF2|PHI:1043|MgHog1|1047171|Zymoseptoria_tritici| loss_of_pathogenicity	81.4	0	78

Table continued on next page.

Table 3C: Hits to the Pathogen-Host Interactions Database by *P. membranifaciens* (GCA_001661235) (cont'd)

Query	Subject|Gene ID|PhiBase ID| Gene|Pathogen ID|Species|Phenotype	%identity	evalue	Query coverage %
ODQ49439.1	I1RA52|PHI:445|NOS1|5518|Fusarium_graminearum| reduced_virulence	81.36	0	79
ODQ47003.1	Q59KY8|PHI:378|SIT4|5476|Candida_albicans| reduced_virulence	81.27	0	100
ODQ44749.1	I1RWQ2|PHI:1235|FGSG_08731|5518| Fusarium_graminearum|lethal	81.02	0	74
ODQ44971.1	Q56R42|PHI:497|HOG1|5207|Cryptococcus_neoformans| reduced_virulence	80.94	0	78
ODQ44971.1	E9EYM7|PHI:5427|Mero-Hog1|568076|Metarhizium_robertsii| reduced_virulence	80.9	0	80
ODQ44971.1	Q92207|PHI:149|HOG1|5476|Candida_albicans| reduced_virulence	80.74	0	80
ODQ45444.1	P02829|PHI:463|HSP90|4932|Saccharomyces_cerevisiae| increased_virulence_(hypervirulence)	80.34	0	99
ODQ48751.1	G4NCL5|PHI:877|MGG_00383|318829|Magnaporthe_oryzae| reduced_virulence	80.26	0	99

All relevant files can be found on the Ascus Drive (b) (4)

Within that directory the database files are found at:
(b) (4)

The assembled Dairy-21 genome can be found at (b) (4)

Results for *Candida krusei / Pichia kudriavzevii* (GCA_003054445) can be found at:

(b) (4)

Files:

(b) (4)

Files:

(b) (4)

Results for *Pichia membranifaciens* (GCA_001661235) can be found at:

(b) (4)

Files:

(b) (4)

(b) (4)

Objectives

The objective of this work was to assess the genome of *Pichia kudriavzevii* ASCUSDY21 for plasmids.

Methods

To detect novel plasmids in the draft genome assembly, the de novo assembly graphs were analyzed (Rozov et al., 2017). Additionally, known fungal plasmids were obtained from Joint Genome Institute (JGI) Integrated Microbial Genomes and Microbes (IMG/M) (Markowitz et al., 2012) and National Center for Biotechnology Information (NCBI) (Agarwala et al., 2018). All of the fungal plasmid sequences were concatenated and used as the reference database. *P. kudriavzevii* ASCUSDY21 was sequenced using an Illumina MiSeq (pair-ended, 2 x 300 cycles). A total number of 2,149,302,600 bases were mapped onto the fungal plasmid reference database using Burrows-Wheeler Aligner (BWA) (Li, 2013; Li & Durbin, 2009).

Results

Two partial contigs were identified as having putative cycle-based arcs in the underlying *de Bruijn* graph. The two contigs were used to query the NCBI nr/nt database. Both contigs aligned to chromosomal segments of closed *Pichia kudriavzevii* genomes, suggesting that they are chromosomal in *P. kudriavzevii* ASCUSDY21 and not plasmid-based.

To further this analysis, the reads generated from sequencing *P. kudriavzevii* ASCUSDY21 were aligned to all known fungal plasmid sequences in the NCBI and IMG/M databases. Of the 2,149,302,600 bases from *P. kudriavzevii* ASCUSDY21, only 107 total bases of known fungal plasmid sequences were covered, suggesting that *P. kudriavzevii* ASCUSDY21 does not contain known fungal plasmids. The full results including coverage files can be found as listed in the documentation section.

Conclusions

The results suggest that *Pichia kudriavzevii* ASCUSDY21 does not contain any known fungal plasmids, and *de novo* plasmid detection did not suggest the presence of extra-chromosomal DNA in the assembly.

Documentation

The methods, analysis, results, and relevant files for comparison to known fungal plasmids can be found at: (b) (4)

The results of *de novo* plasmid detection can be found on the Ascus Drive at (b) (4)

Signed: (b) (4), (b) (6) Date: 11/18/19

References

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Objective

The objective of this work was to test the genetic stability of *Pichia kudriavzevii* ASCUSDY21, throughout the manufacturing process.

Methods

Genomic DNA was isolated from *P. kudriavzevii* ASCUSDY21 via bead-based lysis using the MoBio PowerViral DNA kit (Carlsbad, CA). Sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA), and the resulting libraries were sequenced on an Illumina Miseq. The draft genome was assembled using SPAdes [version 3.13.0] (Bankevich et al 2012). The open reading frames were predicted through AUGUSTUS using all deposited mRNA sequences for *P. kudriavzevii* in NCBI for training (Stanke and Morgenstern 2005). Average Nucleotide Identity (ANI) was computed using MUMmer (Kurtz et al 2004). Reads were aligned to the reference using bowtie2 (Langmead and Salzberg 2012).

Results

A summary of samples selected for evaluation is presented in Table 1. As shown, samples were selected from the Master Cell Bank (MCB) and two Working Cell Banks (WCB1 and WCB2), as well as End of Fermentation (EOF) and Post Drying (PBV) from 3 independent runs (062819, 093019, and 100119) with a pre-drying sample from one of the runs (PM from 062819).

Table 1: Summary of samples and similarity to Master Cell Bank

Sample	Summary	Average Nucleotide Identity (ANI %)
MCB	Master Cell Bank	100.00%
WCB1	Working Cell Bank 1	99.96%
WCB2	Working Cell Bank 2	99.93%
EOF-062819	End of fermentation from a production-scale run	99.97%
EOF-093019	End of fermentation from a lab-scale run	99.97%
EOF-100119	End of fermentation from a lab-scale run	99.97%
PM-062819	Preservation mixture from a production-scale run	99.98%
PBV-062819	Post-drying sample from a production-scale run	99.97%
PBV-093019	Post-drying sample from a lab-scale run	99.97%
PBV-100119	Post-drying sample from a lab-scale run	99.97%

As indicated in Table 1, each sample aligned to the Master Cell Bank reference genome at a rate greater than 99.9%, indicating very little genetic drift during the manufacturing process. This is as expected, considering that a typical seed of 10^6 CFU must undergo 40 generations to reach 10^{18} CFU (a high titer production target at >100,000 L), and at a spontaneous mutation rate of 0.003 per genome (Drake et al 1991), less than 15% of all cells will incur any genetic change from cell bank to end of production.

Genes were identified and annotated in each assembly in order to determine whether pathogenic or resistance genes were acquired or changed at any stage of the manufacturing process. Furthermore, the unmapped reads from each assembly were assembled separately and assessed for gene content. A summary of newly discovered genes is presented in Table 2.

Table 2: Genes not initially found in reference genome

Sample-gene	Description
PBV-100119-g1107.t1	Assembly Error
EOF-062819-g1244.t1	Assembly Error
PM-062819-g189.t1	Assembly Error
PBV-062819-g2301.t1	Assembly Error
PBV-100119-g2498.t1	Assembly Error
PBV-093019-g861.t1	Assembly Error
EOF-100119-g2273.t1	Uncalled gene in genome (non-pathogenic, MAP kinase)
PM-062819-g2311.t1	Uncalled gene in genome (non-pathogenic, MAP kinase)
EOF-093019-g2325.t1	Uncalled gene in genome (non-pathogenic, MAP kinase)
PBV-093019-g2241.t1	Uncalled gene in genome (non-pathogenic, MAP kinase)
PBV-100119-g2246.t1	Uncalled gene in genome (non-pathogenic, MAP kinase)
EOF-093019-g1563.t1	Uncalled gene in genome (non-pathogenic, DOT6 transcriptional regulatory protein)
EOF-100119-g1598.t1	Uncalled gene in genome (non-pathogenic, DOT6 transcriptional regulatory protein)
PBV-093019-g1883.t1	Uncalled gene in genome (non-pathogenic, DOT6 transcriptional regulatory protein)
PBV-100119-g1907.t1	Uncalled gene in genome (non-pathogenic, DOT6 transcriptional regulatory protein)
MCB-g782.t1	Uncalled gene in genome (non-pathogenic, DOT6 transcriptional regulatory protein)
EOF-062819-g1607.t1	Uncalled gene in genome (non-pathogenic, hypothetical protein)
PBV-062819-g2616.t1	Uncalled gene in genome (non-pathogenic, hypothetical protein)
EOF-062819-g1301.t1	Uncalled gene in genome (non-pathogenic, protein ligase)

6 of the discovered genes were attributed to assembly errors, with stretches of greater than 20 homopolymer repeats and no homologous proteins found in the NCBI database. The remaining 13 genes were found in the reference genome upon searching the nucleotide sequence but were not detected in the initial annotation by the gene-finding algorithm. In fact, only 5 new genes were discovered, but were detected in up to 5 distinct resequencing samples.

Conclusions

When sampled from three separate runs at various stages of processing, the genome of *P. kudriavzevii* ASCUSDY21 did not significantly change and no new pathogenic, resistant, or virulent genes resulted from processing. Consequently, the production process of *P. kudriavzevii* ASCUSDY21 is unlikely to result in changes to the genetic assessment of safety.

Documentation

The data for this analysis can be found on the Ascus Drive under (b) (4)

(b) (4), (b) (6)

Signed: __________ Date: 11/18/19

References

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Drake, J.W. (1991). A constant rate of spontaneous mutation in DNA-based microbes. *Proceedings of the National Academy of Science USA.* 88: 7160–7164.

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Mannitol

(b) (4)

Specification for Mannitol, Powder, USP (MA165)

Item Number	MA165
Item	Mannitol, Powder, USP
CAS Number	69-65-8
Molecular Formula	$C_6H_{14}O_6$
Molecular Weight	182.17
MDL Number	
Synonyms	Cordycepic Acid ; 1,2,3,4,5,6-Hexanehexol ; Mannite ; D-Mannitol

Test	Specification	
	Min	Max
ASSAY (DRIED BASIS)	97.0	102.0 %
MELTING RANGE	165° -170°C	
APPEARANCE OF SOLUTION	TO PASS TEST	
LOSS ON DRYING		0.5 %
NICKEL		1 µg/g
CONDUCTIVITY @ 25 C		20 µS/cm
RELATED SUBSTANCES	TO PASS TEST	
REDUCING SUGARS	TO PASS TEST	
MICROBIAL LIMITS:		
TOTAL AEROBIC MICROBIAL COUNT		10^3 cfu/g
TOTAL COMBINED MOLDS AND YEASTS COUNT		10^2 cfu/g
ESCHERICHIA COLI		Negative
ELEMENTAL IMPURITIES	AS REPORTED	
IDENTIFICATION	TO PASS TEST	
EXPIRATION DATE		
DATE OF MANUFACTURE		
APPEARANCE		
RESIDUAL SOLVENTS	TO PASS TEST	

(b) (4)

Sucrose

(b) (4)

Product Use and Information

Fine/Extra Fine Granulated Sugar

Fine/Extra Fine Granulated Sugar, a food grade product, is made by crystallizing a purified and filtered thick juice syrup removed from sugar beets or sugar cane, which is then dried and screened to produce the most popular sugar grades. It is white in color and has sucrose content of not less than 99.85 percent. This product is either referred to as Fine Granulated Sugar or Extra Fine Granulated Sugar depending on local market.

Applications
Pharmaceuticals, jams, jellies, meats, chewing gum, dairy products, condiments, pickles, bakery products, cereals, liquid sugar, powdered sugar, candies and powdered drink mixes.

Products
Fine Granulated Sugar is available in bulk rail and bulk trucks, as well as 2, 4, 5, 10, 25, 50 pound bags, totes and supersacks.

General Requirements
This food grade product is manufactured in accordance with Current Good Manufacturing Practices and complies with the Federal Food Drug and Cosmetic Act and all other FDA regulations as well as any applicable state statutes and regulations.

Characteristics

Sucrose	NLT* 99.85%
Sediment	NMT** 3 ppm
Ash	NMT** 0.025%
Moisture	NMT** 0.035%
Color	NMT** 45 RBU/IU
Invert	NMT** 0.050%
Sulfite	NMT** 10 ppm as SO_2
Speck Count (visual)	NMT** 2 per 500 grams
Odor	Free of foreign odors

Granulation

U.S. Sieve #	Maximum
20	2.0% cumulative retained
100	5.0% passing

Microbiological Standards
- Product shall test negative for pathogenic microorganisms.
- May also be ordered to meet and National Soft Drink Association requirements.

* NLT - No less than.
** NMT - No more than.

This product information sheet is correct to the best of our knowledge. However, the information, recommendations and suggestions are made without representation or guarantee as to results because the conditions of use of the product are beyond our control and accordingly are furnished only for your consideration, investigation and verification by your own laboratory prior to use. No statement is to be construed as a waiver of any copyright or patent right.

Revised June, 2018

(b) (4)

27 Stearine

(b) (4)

Product Data Sheet

Product Description:
27 Stearine is a highly functional hardened palm oil. Palm stearines crystallize into a stable betaprime configuration. Beta-prime hard fats crystallize into permanent fine grained crystals. This allows for maximum oil stabilization as well as stability over a broad range of storage conditions.

Typical data suggests that it may be used for stabilizing peanut butter, as well as a melt point adjuster for many types of processed foods. The user is advised to fully evaluate the functionality and shelf life of the shortening in their intended finished product at their own facilities, as performance may be affected by varying formulations and process conditions.

Ingredient Statement:
Hydrogenated Palm Oil. Kosher. (US)

Typical Data:

Capillary Melting Point	.......58-62ºC/136-144ºF
Color (5 1/4") Lovibond	.......5 R max
Free Fatty Acid (% as oleic)	.......0.10% max
Iodine Value	.......4 max

The typical data provided here is valid at the point of shipment from our manufacturing facility.

Packaging:
27 Stearine is available in 50 lb. beaded poly-lined cartons and in bulk liquid.

Storage and Handling:
27 Stearine needs no refrigeration, however, like all fats, it will absorb odors and should be stored between 40-80ºF in a dry place away from odor-producing substances. Bulk liquid product can be stored at 150-160ºF for 30 days. Based on the typical data a shelf-life of 180 days is suggested for packaged product stored at 40-80ºF. *

Service:
A sales representative will be pleased to assist you in the use of this product. For additional information technical support or service, please call Loders Croklaan at 800-621-4710.

(b) (4)

* Actual shelf-life may vary and is dependant upon several factors including the type of application, interaction with other components of the finished product, process conditions used in the preparation of the finished product and conditions of subsequent storage and shipping of the finished product. The user is advised to carry out a full evaluation of the shortening to determine its suitability in their finished product.

This information is presented for your consideration in the belief that it is accurate and reliable; however, no guarantee or warranty either expressed or implied is made since conditions of use are beyond our control and no freedom from liability from patents, trademarks, or other limitations should be inferred.

(b) (4)

Rev. date 06/09

ASCUS BIOSCIENCES

Specifications for Sodium Sulfate

Ingredient:	Sodium Sulfate
Chemical Nomenclature:	$NaSO_4$
Specifications:	Feed Grade
Moisture:	≤ 1% by LOD
Purity:	≥ 98%

Ammonia Solution

(b) (4)

AMMONIA SOLUTION FG

PRODUCT DESCRIPTION

Ammonia Solution FG is a water white solution of anhydrous ammonia in water that meets food grade requirements and reference 21 CFR 184.1139.

CAS No. 1336-21-6

SYNONYMS

Ammonium hydrate

USES AND APPLICATIONS

Ammonia Solution FG may be used to replace anhydrous ammonia in most applications.

Product is designed for use in food and fermentation applications.

TYPICAL VALUES

Appearance:	Clear, Colorless Liquid
Odor:	Ammonia
Ammonia as NH_3 (wt.%):	27.0 – 30.0
Lead (ppm):	0.5 max
Nonvolatile Reside (wt.%):	0.02 max
Readily Oxidizable Substances:	Passes FCC Test

SHELF LIFE: Shelf life stability is dependent on temperature. At room temperature, the shelf life is 90 days.

PRECAUTIONS

Product safety information and handling precautions are contained on the product label and Safety Data Sheet (SDS).

READ AND UNDERSTAND LABEL AND SAFETY DATA SHEET BEFORE PRODUCT USE.

(b) (4)

Ammonium sulfate

AMMONIUM SULFATE $(NH_4)_2SO_4$

Specifications

FCC GRANULAR GRADE

Identification - Ammonium and Sulfate	Passes Tests
Assay-Ammonium Sulfate	99.0-100.5 %
Water Insoluble	≤ 0.3 %
Residue On Ignition	≤ 0.25 %
Moisture	≤ 0.15 %
Iron	≤ 15 ppm
Selenium	≤ 5 ppm
Lead	≤ 3 ppm
Arsenic	≤ 0.5 ppm

This information is for the specific material described **only** and may not be valid if the material is used in combination with any other material or in any process. The user is responsible to determine the completeness of the information and suitability for the user's own particular use. To the knowledge and

(b) (4)

Issued: 06/23/15
Last Reviewed: 10/25/18

Biotin

(b) (4)

Specification for Biotin, Powder, FCC (BI115)

Item Number	BI115
Item	Biotin, Powder, FCC
CAS Number	58-85-5
Molecular Formula	$C_{10}H_{16}N_2O_3S$
Molecular Weight	244.31
MDL Number	
Synonyms	Vitamin H

Test	Specification	
	Min	Max
ASSAY ($C_{10}H_{16}N_2O_3S$)	97.5-100.5 %	
MELTING RANGE	229 - 232 C (dec	
OPTICAL ROTATION	+89 to+93	
LEAD (Pb)		2 mg/kg
IDENTIFICATION		TO PASS TEST
RETEST DATE		

(b) (4)

Calcium Chloride, Dihydrate

(b) (4)

Specification for Calcium Chloride, Dihydrate, USP, EP, BP, JP (CA140)

Item Number	CA140
Item	Calcium Chloride, Dihydrate, USP, EP, BP, JP
CAS Number	10035-04-8
Molecular Formula	$CaCl_2.2H_2O$
Molecular Weight	147.01
MDL Number	
Synonyms	

Test	Specification	
	Min	Max
ASSAY	99.0 - 103.0%	
pH OF A 5% SOLUTION	4.5 - 9.2	
IRON, ALUMINUM, PHOSPHATE	TO PASS TEST	
HEAVY METALS		0.001%
MAGNESIUM AND ALKALI SALTS		0.5%
APPEARANCE OF SOLUTION	TO PASS TEST	
SULFATE		0.024%
HYPOCHLORITE	TO PASS TEST	
BARIUM	TO PASS TEST	
ARSENIC (As)		2 ppm
ACIDITY / ALKALINITY	TO PASS TEST	
ALUMINUM	TO PASS TEST	
IRON (EP)		10 ppm
IDENTIFICATION	TO PASS TEST	
RETEST DATE		
RESIDUAL SOLVENTS	TO PASS TEST	

(b) (4)

Calcium Chloride, Dihydrate

(b) (4)

Specification for Calcium Chloride, Dihydrate, USP, EP, BP, JP (CA140)

Item Number	CA140
Item	Calcium Chloride, Dihydrate, USP, EP, BP, JP
CAS Number	10035-04-8
Molecular Formula	$CaCl_2.2H_2O$
Molecular Weight	147.01
MDL Number	
Synonyms	

Test	Specification	
	Min	Max
ASSAY	99.0 - 103.0%	
pH OF A 5% SOLUTION	4.5 - 9.2	
IRON, ALUMINUM, PHOSPHATE	TO PASS TEST	
HEAVY METALS		0.001%
MAGNESIUM AND ALKALI SALTS		0.5%
APPEARANCE OF SOLUTION	TO PASS TEST	
SULFATE		0.024%
HYPOCHLORITE	TO PASS TEST	
BARIUM	TO PASS TEST	
ARSENIC (As)		2 ppm
ACIDITY / ALKALINITY	TO PASS TEST	
ALUMINUM	TO PASS TEST	
IRON (EP)		10 ppm
IDENTIFICATION	TO PASS TEST	
RETEST DATE		
RESIDUAL SOLVENTS	TO PASS TEST	

(b) (4)

Cerelose® Dextrose M Non-GMO

technical specification

(b) (4)

CERELOSE® Dextrose M NON-GMO IP 02001090

CERELOSE® Dextrose 02001090 is a general purpose crystalline monohydrate dextrose suitable for most food, beverage, and industrial uses. This product is produced under Ingredion Incorporated's TrueTrace™ Program for non-GM products.

Chemical and Physical Properties

	Min.	Max.
Moisture %	8.0	9.0
Dextrose Equivalent	99.5	-
SO_2, ppm	-	<10
Dextrose, % d.b.	99.5	-
Ash, % d.b.	-	0.1
Solution Color		Passes test
Apparent Starch		Passes test

Physical Appearance

	Typical
Color	White
Form	Powder

Screen Test

	Typical
On USS 20 mesh, %	<1
On USS 100 mesh, %	<60

Microbiological Limits

	Max.
Standard Plate Count, cfu/g	100
Yeast, cfu/g	25
Mold, cfu/g	25
Salmonella/10 g	Negative
Coliforms, MPN/g	3

Nutritional Data/ 100g

	Typical
Calories	362
Calories from Fat	0
Total Fat, g	0
Cholesterol, mg	0
Sodium, mg	0
Total Carbohydrate, g	90.5
Dietary Fiber, g	0
Total Sugars**, g	90.5
Added Sugars, g	0
Other Carbohydrate, g	0
Protein, g	0
Vitamin D, mcg	0
Calcium mg	0
Iron, mg	0
Potassium, mg	0
Ash, g	<0.1*

* Not present at level of quantification.
** "Total Sugars" in this product may contribute to "Added Sugars" for nutrition labeling purposes in the final consumer product.

Certification

Kosher Pareve
Halal

Packaging and Storage

Bags
Product should be stored in a clean, dry area, not exposed to prolonged high (> 90°F / 32°C) temperature.

Shelf Life

3 years, provided product is stored the original container, well-closed in a cool, dried place free from humidity, dust, or foreign contamination.

Regulatory Data

Source	Corn (IP-TrueTrace™)
CAS No.	50-99-7

United States

Meets FCC (Food Chemical Codex) requirements.

Standard of Identity	21 CFR 168.111
GRAS Affirmation	21 CFR 184.1857
Labeling	Dextrose or Dextrose monohydrate

Canada

Standard Food	CFDA Regulation
Standard of Identity	B.18.015
Labeling	Dextrose or Dextrose monohydrate

Features and Benefits

TrueTrace™ certified non-GM.
Dry crystalline powder, Free flowing.
Mild sweetness
Bulking, Carrying
Highly fermentable

(b) (4)

Copper Sulfate Pentahydrate

(b) (4)

Cupric Sulfate, 5-Hydrate
U.S.P.

Product No. 1844
Specifications current as of: Mar 21 2019

TEST	SPECIFICATION
GMP Manufactured Product	
Meets U.S.P Requirements	
CAUTION: For Manufacturing, processing or repackaging	
Bulk Pharmaceutical Chemical	
USP - Calcium (Ca)	<= 0.005 %
USP - Identification	Passes Test
USP - Iron (Fe)	<= 0.003 %
USP - Loss on Drying	33.0 - 36.5 %
USP - Nickel (Ni)	<= 0.005 %
USP - Potassium (K)	<= 0.01 %
USP - Sodium (Na)	<= 0.02 %
USP - Assay ($CuSO_4$) (dried basis)	98.5 - 100.5 %

No Class 1,2,3 or other solvents are used or produced in the manufacturing or purification of the product.

(b) (4)

Dipotassium Phosphate

(b) (4)

(b) (4)

DIPOTASSIUM PHOSPHATE
FOOD GRADE - EC/FCC

DESCRIPTION	Dipotassium Phosphate is a white, granular or milled product, which is essentially odorless. It is deliquescent with exposure to moist air.
USES	Food √ Buffering agent for processing foods √ Stabilizer for non-dairy coffee creamers Pharmaceuticals √ Nutrient for antibiotic production √ Humectant
NOMENCLATURE	Potassium Phosphate, Dibasic Dipotassium Monohydrogen Orthophosphate
FORMULA	K_2HPO_4
FORMULA WEIGHT	174.2
REFERENCE NUMBERS	CAS# 7758-11-4, E-340(ii)
CAS INDEX NAME	Phosphoric Acid, Dipotassium Salt
STORAGE	Store at Room Temperature
RE-TEST DATE	24 months after the date of manufacture
CERTIFICATES	Includes Kosher, NAFTA, NSF, HALAL and others.
LABEL DECLARATION	Potassium Phosphate
GRADE	**COMPLIES WITH FOOD CHEMICALS CODEX AND EUROPEAN COMMUNITY REQUIREMENTS.**

(b) (4)

All information is offered in good faith, without guarantee or obligation for the accuracy or sufficiency thereof, or the results obtained, and is accepted at user's risk. Nothing herein shall be construed as a recommendation for uses which infringe valid patents or as extending license under valid patents.

(b) (4)

DIPOTASSIUM PHOSPHATE
FOOD GRADE EC/FCC GRADE

SHIPPING POINTS (b) (4)

CONTAINERS 50-pound net weight plastic bags (40 bags per pallet)
25-kg net weight plastic bags (40 bags per pallet)

SPECIFICATIONS

Assay (K_2HPO_4, dry basis)	98.0% min.
Phosphate as P2O5	40.3 – 41.5 %
pH, 1 % solution	8.7 – 9.4
Loss on drying	2.0 % max.
Water insoluble	0.2 % max.
Fluoride as F	10 ppm max.
Arsenic as As	1 ppm max.
Lead as Pb	1 ppm max.
Cadmium as Cd	1 ppm max.
Mercury as Hg	1 ppm max.
Identification Phosphate	Pass
Identification Potassium	Pass

TYPICAL PROPERTIES

Sieving	Available as granular or milled product
On 10 mesh	Trace
Bulk Density (g/cc)	1.03
Solubility	Approx. 63 grams per 100 grams saturated solution in 25°C.

2/3/15

(b) (4)

All information is offered in good faith, without guarantee or obligation for the accuracy or sufficiency thereof, or the results obtained, and is accepted at user's risk. Nothing herein shall be construed as a recommendation for uses which infringe valid patents or as extending license under valid patents.

Dystar KFO-402 Antifoam

(b) (4)

KFO™ 402

Product Type	**FOOD GRADE – GENERAL PURPOSE PROCESS AID DEFOAMER**
Product Description	KFO™ 402 is a defoamer designated to control foam in many processes. KFO™ 402 is especially effective when used in fermentation processes where a certain degree of foam control is needed without affecting oxygen transfer for optimum product yield. This product is made with food grade ingredients under our Good Manufacturing Practices Program. The components of KFO™ 402 meet FDA requirement for use in egg washing, potato processing defoamers as a dispersing aid for mineral oil at a limit of 10 ppm in the processing water followed by a potable water rinse. This product also contains ingredients for which the FDA has provided the Enzyme Technical Association with a "no objection" letter acknowledging that they are used as defoaming agents in the manufacture of enzyme preparations used in food in accordance with the principles of GMPs. Other uses in the processing and manufacture of food ingredients may also qualify for GRAS status. KFO™ 402 also is composed of ingredients that meet the current requirements of the FDA for food contact applications when used in accordance with the requirements and limitations of 21CFR 176.210(d)(3). Consideration for other FDA permitted uses would require further evaluation.

Typical Properties

Property	Value
Appearance	Clear Liquid
Viscosity @ 100°F, Kinematic	185 – 210 Cst
Odor	Sweet
Weight per gallon	8.5 Lbs
Flash Point (°C)	> 216°C PMCC (Min)
Specific Gravity	1.02

Typical Applications	Typical applications for KFO™ 402 include: • Fermentation • Egg washing
Incorporation	KFO™ 402 should be added, as received, early in the processing to prevent foam before it forms. KFO™ 402 should be evaluated in the process to determine the optimum dosage and legal limits allowed.
Shelf Life	2 years from date of manufacture when properly stored in the original container following proper storage and handling.
Storage & Handling	Keep from freezing. Store product between 40 and 100°F. Keep containers tightly closed when not in use.
Responsible Care	For complete safety, health, personnel protection and first aid information, refer to the Safety Data Sheet (SDS) that can be ordered through the numbers below.

Updated January 16, 2017

(b) (4)

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

September 11, 2003

(b) (4)

Dear Mr. (b) (4):

You requested, on behalf of the Enzyme Technical Association, that OFAS review the use of certain defoaming and flocculating agents in the manufacture of enzyme preparations used in food. You provided information related to these compounds in your letters of December 20, 1996 (to Dr. Alan Rulis), 4-24-1998 (to Dr. Zofia Olempska-Beer), and 11-30-99 (to Dr. Zofia Olempska-Beer). You also arranged for a teleconference between ETA members and OFAS representatives, facilitated telephone contacts with technical experts from ETA member companies, and responded to numerous requests for clarification. We appreciate your and ETA's cooperation.

We reviewed the information on defoaming and flocculating agents that you submitted as well as the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. The enclosed attachment provides a brief overview of our evaluation and itemizes the evaluated defoamers (Table 1) and flocculants (Table 2). We conclude that these compounds are used by enzyme manufacturers in accordance with the principles of good manufacturing practice (GMP).

Sincerely yours,

Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition

Defoaming and Flocculating Agents Used in the Manufacture of Enzyme Preparations Used in Food

Enzyme Preparations

Most enzymes currently used in food are derived from microorganisms. The manufacturing process of such enzymes includes three major steps: fermentation, enzyme recovery, and enzyme formulation. The formulated products are generally referred to as enzyme preparations. In addition to the enzymes of interest, enzyme preparations contain added substances such as diluents, preservatives, and stabilizers. They may also contain metabolites derived from the production microorganism and the residues of substances used in the manufacturing process, such as components of the fermentation medium or defoaming and flocculating agents used during fermentation and recovery. When FDA reviews safety data on enzyme preparations, it considers all components of the preparation.

Defoaming Agents

Defoaming agents (defoamers) are used by enzyme manufacturers to reduce or prevent foaming during fermentation and recovery. They are formulated with ancillary ingredients such as surface-active agents or carriers. Defoamers currently used in the manufacture of food enzymes are listed in Table 1. The Table includes five major defoamers that are identified by a double asterisk and several compounds that are used either as secondary defoamers or ancillary ingredients in defoamer formulations.

The major defoamers are added to the fermentation broth at levels within the range of 0.05-1% on a weight basis. Some of these defoamers, for example, polyoxyethylene-polyoxypropylene block copolymer, may contain trace levels of ethylene oxide, propylene oxide, and 1,4-dioxane which are known to cause cancer in laboratory animals. The Office of Food Additive Safety (OFAS) has evaluated the use of defoamers listed in Table 1 and determined that human exposure to the residues of these defoamers in enzyme preparations does not present human safety concern.

Flocculating Agents

Flocculating agents (flocculants) are used in the enzyme recovery step to separate microbial cells and cell debris from the fermentation broth containing the dissolved enzyme. The flocculation typically consists of two steps - primary flocculation and secondary flocculation. In the primary flocculation, inorganic salts (such as calcium chloride or aluminum sulfate) or "low molecular weight" polymers (such as polyamines) are used to agglomerate the cellular debris. The primary flocculation is usually followed by the secondary flocculation in which "high molecular weight" polymers are used to aid the formation of larger agglomerates that are subsequently removed by centrifugation or filtration. The polymers used as flocculants can be either cationic or anionic. The cationic polymers are added to the fermentation broth at levels not higher than 1% on a

weight basis. The anionic polymers are used at levels at or below 0.025%.

The flocculants used in the manufacture of food enzymes are listed in Table 2. They include inorganic salts, polyamines, and polyacrylamides. Several of these compounds are regulated in 21 CFR either as food additives or GRAS substances. Certain polyamines may contain traces of epichlorohydrin and 1,3-dichloro-2-propanol. Polyacrylamides usually contain very low levels of acrylamide. These contaminants of polyamines and polyacrylamides are known to cause cancer in laboratory animals. OFAS has evaluated all polymers included in Table 2 and determined that human exposure to the residues of these flocculants in enzyme preparations does not present human safety concern.

Sources of Information on Defoamers and Flocculants

OFAS compiled data on defoamers and flocculants listed in Tables 1 and 2 using information voluntarily submitted by the Enzyme Technical Association. OFAS also relied on the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. Other sources of information included published articles, computer searches, and Material Safety Data Sheets issued by manufacturers of defoamers and flocculants.

Table 1. Defoamers Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Polypropylene glycol**	25322-69-4	Average MW: 2000
Polyglycerol polyethylene-polypropylene glycol ether oleate**	78041-14-2	
Polyoxyethylene-polyoxypropylene block copolymer**	9003-11-6	Average MW: 2000
Polypropylene glycol monobutyl ether**	9003-13-8	
Polydimethylsiloxane**	63148-62-9 68083-18-1	
Silica	7631-86-9 63231-67-4	
Stearic acid	57-11-4	
Sorbitan sesquioleate	8007-43-0	
Glycerol monostearate	123-94-4	
Polysorbates (polyoxyethylene sorbitan fatty acid esters)		Polysorbate 60 (CAS No. 9005-67-8), Polysorbate 65 (CAS No. 9005-71-4), and polysorbate 80 (CAS No. 9005-65-6) are regulated as food additives and components of defoamer formulations
Rape oil mono- and diglycerides	93763-31-6	
White mineral oil	64742-47-8	

Table 2. Flocculants Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Dimethylamine-epichlorohydrin copolymer	25988-97-0	Cationic polyamine
Methylamine-epichlorohydrin copolymer	31568-35-1	Cationic polyamine
Dimethylamine-epichlorohydrin-ethylenediamine terpolymer	42751-79-1	Cationic polyamine
Polyacrylamide modified by condensation with formaldehyde and dimethylamine	67953-80-4	Cationic polyacrylamide
Acrylamide-acryloxyethyl-trimethyl-ammonium chloride copolymer	69418-26-4	Cationic polyacrylamide
Acrylamide-acrylic acid copolymer	25987-30-8 9003-06-9	Anionic polyacrylamide
Aluminum sulfate	10043-01-3	
Calcium chloride	10035-04-8 10043-52-4	

Ferric Ammonium Citrate

(b) (4)

Specification for Ferric Ammonium Citrate, Brown, Powder, FCC (F1000)

Item Number	F1000
Item	Ferric Ammonium Citrate, Brown, Powder, FCC
CAS Number	1185-57-5
Molecular Formula	
Molecular Weight	
MDL Number	
Synonyms	Iron Ammonium Citrate

Test	Specification	
	Min	Max
ASSAY (Fe)	16.5 - 18.5 %	
FERRIC CITRATE		TO PASS TEST
OXALATE (C_2O_4)		TO PASS TEST
LEAD (Pb)		2 mg/kg
MERCURY		1 mg/kg
SULFATE		0.3 %
IDENTIFICATION		TO PASS TEST
RETEST DATE		

(b) (4)

Hydrogen Chloride 1M

(b) (4)

Specification for Hydrochloric Acid, 37 Percent, FCC (HY106)

Item Number	HY106
Item	Hydrochloric Acid, 37 Percent, FCC
CAS Number	7647-01-0
Molecular Formula	HCl
Molecular Weight	36.46
MDL Number	
Synonyms	Chlorhydric Acid ; Muriatic Acid

Test	Specification	
	Min	Max
ASSAY	36.0 - 38.0 %	
COLOR		TO PASS TEST
SPECIFIC GRAVITY		TO PASS TEST
IRON (Fe)		5 mg/kg
LEAD (Pb)		1 mg/kg
MERCURY		0.10 mg/kg
NONVOLATILE RESIDUE		0.5 %
ORGANIC COMPOUNDS		TO PASS TEST
OXIDIZING SUBSTANCES (as Cl2)		0.003 %
REDUCING SUBSTANCES (as SO_3)		0.007 %
SULFATE		0.5 %
IDENTIFICATION		TO PASS TEST
EXPIRATION DATE		
DATE OF MANUFACTURE		
APPEARANCE		

(b) (4)

Magnesium Sulfate Heptahydrate

(b) (4)

(b) (4)

SALES SPECIFICATIONS FOR
MAGNESIUM SULFATE CRYSTALS, USP
DECEMBER 1ST, 2018

CHARACTERISTICS

The material shall be colorless, solid at ambient temperatures, formed in small needle-like rhombic crystals and free of solid or fibrous foreign matter that will require the dissolved material to be filtered before being used. Epsom salt is one of the most common forms of magnesium sulfate heptahydrate. EPSOM SALT is a hydrated salt with seven molecules of water, so caking or bridging can occur. Care should be taken to protect the material if it is stored in the granular form for long periods of time. EPSOM SALT is readily soluble in water.

PROPERTIES			
	Identification	Test of Magnesium	Positive
		Test of Sulfate	Positive
		Consistently Free of Volatile Organic Impurities	
	Chemical	pH	5.0 - 9.2
		Loss of ignition	40.0% - 52.0%
		Chloride, maximum, ppm	140
		Arsenic, maximum, ppm	3
		Heavy Metals, maximum, ppm	10
		Iron, maximum, ppm	20
		Selenium, maximum, ppm	30
		Assay	99%-100.5%
	Physical	Color	Colorless
		Crystal Form	Rhombic (monoclinic)

QUALITY ASSURANCE PROVISION

GENERAL:

The material specified herein shall be manufactured using acceptable industrial practices.
The material shall be guaranteed to meet chemical and physical properties specified herein.

RESPONSIBILITIES FOR TESTS & INSPECTIONS:

Unless otherwise specified by purchaser, the supplier is responsible for providing a lot analysis on the material. Except as otherwise specified, the supplier may utilize his own facilities or any commercial laboratory. Analysis' are available for each lot at an additional charge.

PACKAGING & SHIPPING

PACKAGING:

Shall be accomplished in accordance with acceptable commercial practices for domestic or foreign shipments unless otherwise indicated by the purchaser. It shall be the vendor's responsibility to determine that packaging, as done, is adequate to assure that all materials shall arrive at destination in an uncontaminated condition and ready for intended use.

SHIPPING:

Shall be accomplished in accordance with acceptable commercial practices for domestic or foreign shipment for this type of product unless otherwise indicated by the purchaser.

(b) (4)

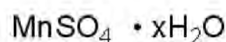
Manganese Sulfate, Monohydrate

(b) (4)

Product Specification

Product Name:
Manganese sulfate monohydrate – meets USP testing specifications

Product Number:	**M8179**
CAS Number:	10034-96-5
MDL:	MFCD00149159
Formula:	MnO4S · H2O
Formula Weight:	169.02 g/mol



TEST	Specification
Assay	98.0 - 102.0 %
Identity	Pass
Loss on Ignition	10.0 - 13.0 %
Substances not ppt. by (NH4)2S	≤ 0.5 %
Residual Solvents USP 467	Meets Requirements
Recommended Retest Period 6 Years	----------------

Specification: PRD.0.ZQ5.10000033488

(b) (4) warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at (b) (4) For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Monopotassium phosphate (MKP)

(b) (4)

(b) (4)

MONOPOTASSIUM PHOSPHATE (MKP)

GRADE: FCC
Complies with the specifications of the current edition of the Food Chemicals Codex

DESCRIPTION: White, odorless, crystalline material

NOMENCLATURE: Potassium Dihydrogen Phosphate
Potassium Phosphate, Monobasic

FORMULA: KH_2PO_4

FORMULA WEIGHT: 136.1

CAS NUMBER: 7778-77-0

E/INS NUMBER: 340(i)

SPECIFICATIONS[1]:

FCC Specifications		
Assay (KH_2PO_4)	NLT* 98.0	%
Arsenic (As)	NMT** 3 (Max 3)†	mg/kg (ppm)
Fluoride (F)	NMT 10 (Max 10)	mg/kg (ppm)
Lead (Pb)	NMT 2 (Max 2)	mg/kg (ppm)
Insoluble Substances	NMT 0.2	%
Loss on Drying	NMT 1	%
Innophos Specifications		
Assay - P_2O_5 Content	51.0 – 53.0	%
pH (1% solution)	4.2 – 4.7	
Sieving On 10 mesh	0 max	%
Sieving On 100 mesh	80 min	%

*not less than **not more than † values in parenthesis are in format as they appear on the Certificate of Analysis (COA)

[1] These specifications may not all be reported values on Certificates of Analysis for this product. In addition, some specifications and values reported on Certificates of Analysis may be based on periodic testing, not lot release testing. Microbial testing is not performed on this product. It is manufactured according to good manufacturing practices meeting food regulations in the place of manufacture, including physical, chemical and microbial hazard assessments.

(b) (4)

All information is offered in good faith, without guarantee or obligation for its accuracy or sufficiency or for the results a user obtains, and is at the user's risk. User must determine the suitability of the product for its particular use. Nothing herein shall be construed as a recommendation for uses which infringe valid patents or as extending license under valid patents. The current specification may be available a (b) (4)

Effective Date: February 23, 2018
Review Date: February 23, 2020

(b) (4)

(b) (4)

Monopotassium Phosphate (MKP)

Additional Information[2]

pH (1% solution)	4.5
Bulk Density (lbs / ft^3)	74

Uses: Sports beverages, coffee creamers, process cheese products, fortification in food products, etc.

Function: Nutritional source of potassium and phosphorus
Buffer in foodstuffs
Ingredients in emulsifying salts for processed cheese products
Culture nutrients in pharmaceutical manufacture

Certifications: Kosher, NAFTA

Manufacturing Location: (b) (4)

Shipping Points:

Standard Containers: 50-lb net weight plastic bags (40 bags per pallet)

Storage Conditions: Store at room temperature

Retest Date: 730 days from the date of manufacture

2 This section, "Additional Information," is provided for educational purposes only. These values are not reported on the product's Certificate of Analysis.

(b) (4)

All information is offered in good faith, without guarantee or obligation for its accuracy or sufficiency or for the results a user obtains, and is at the user's risk. User must determine the suitability of the product for its particular use. Nothing herein shall be construed as a recommendation for uses which infringe valid patents or as extending license under valid patents. The current specification may be available at (b) (4)

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Safety Evaluation of Monopotassium Phosphate for Use as Mineral Substance for Use in the Production of Direct-Fed Microbials for Use in Animal Feed

ASCUS Biosciences, Inc.

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Safety Evaluation of Monopotassium Phosphate for Use as Mineral Substance for Use in the Production of Direct-Fed Microbials for Use in Animal Feed

1. INTRODUCTION

ASCUS Biosciences, Inc. (hereafter referred to as "ASCUS") develops direct-fed microbial (DFM) products for use as supplementary feeds for poultry and cattle in the United States (U.S.). One of the raw materials used to charge the fermenter for the production of the DFM strains is monopotassium phosphate. While dipotassium phosphate is permitted for use as a sequestrant in feed in accordance with good manufacturing or feeding practice under 21 CFR §582.6282[1], monopotassium phosphate is currently not currently acceptable for feeding to animals in the U.S. Considering that all raw materials used in the production of DFM products should be accepted feed substances in the U.S., ASCUS has conducted a safety evaluation to confirm the suitability of monopotassium phosphate for the intended use as a processing aid in the fermentation of its microbial strains.

2. REGULATORY STATUS

2.1 Regulatory Status in Animal Feed in the U.S.

A number of related phosphate salts are acceptable for use in animal feed in the U.S. and are summarized in Table 2.1.

Table 2.1: Examples of Related Phosphate Salts Accepted for Use in Animal Feed in the U.S.		
Mineral Substance	Function in Feed	Regulatory Status
Diammonium phosphate	Mineral product and general purpose food additive	21 CFR §582.1141 and AAFCO ingredient definition 57.16
Dicalcium phosphate	Mineral product and general purpose food additive	21 CFR §582.1217, 21 CFR §582.5217 and AAFCO ingredient definition 57.71
Disodium phosphate	Mineral product and general purpose food additive	21 CFR §582.1778, 21 CFR §582.5778 and AAFCO ingredient definition 57.32
Monoammonium phosphate	Mineral product and general purpose food additive	21 CFR §582.1141 and AAFCO ingredient definition 57.33
Monocalcium phosphate	Mineral product and general purpose food additive	21 CFR §582.1217, 21 CFR §582.5217 and AAFCO ingredient definition 57.98
Monosodium phosphate	Mineral product and general purpose food additive	21 CFR §582.1778, 21 CFR §582.5778 and AAFCO ingredient definition 57.99
Phosphoric acid	Mineral product and general purpose food additive	21 CFR §582.1073 and AAFCO ingredient definition 57.19
Dipotassium phosphate	Sequestrant	21 CFR §582.6282

[1] https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=582.6285&SearchTerm=dipotassium%20phosphate

2.2 Regulatory Status in Animal Feed in Canada

Monopotassium phosphate is permitted for use in animal feed as in Canada as a Class 6 – Mineral Product under Schedule IV, Part I of the Feed Regulations (1983). The substance must be labeled with guarantees for minimum percent potassium, minimum percent phosphorus and maximum milligrams fluorine, arsenic and iron per kilogram

2.3 Regulatory Status in Animal Feed in the European Union (EU)

Monopotassium phosphate is a recognized feed material in the EU and listed in the Feed Materials Catalogue laid down under Commission Regulation (EU) No 68/2013 (European Commission, 2013). The substance must be labeled with total phosphorus, potassium and, where greater than 10%, the content of phosphorus insoluble in citric acid.

2.4 Regulatory Status in Human Food in the U.S.

Monopotassium phosphate is generally recognized as safe as a food additive in frozen eggs at levels of less than 0.5% in accordance with 21 CFR §160.110.

3. SAFETY EVALUATION FOR TARGET ANIMALS

3.1 History of Use

As mentioned in Section 2, monopotassium phosphate has a long and established history of use as a mineral substance for use in animal feed in Canada and the EU. The levels of monopotassium phosphate as a source of phosphorus in feed is expected to be higher than the residues arising from carry-over of the fermentation process in DFM products. On this basis, the history of safe use of monopotassium phosphate in Canada and the EU for use in animal feed supports the suitability of the additive for use as a raw material in the fermentation of microbial strains by ASCUS.

3.2 Natural Occurrence

Potassium is present in most feedstuffs with the highest levels typically reported in protein sources such as soybean meal. Thus, deficiencies in animals, particularly non ruminants are rare (NRC, 2005). Where diets contain high levels of industrial by-products such as brewer's grains or corn gluten, supplementation can be required.

Likewise, phosphates are widely available from the feed, with oilseed meals and other plant-based materials, mineral feeds, and meat and marine animal feeds serving as major sources in the diet of animals. Availability of phosphorus from the diet can vary with the source and is generally taken into account in the formulation of livestock diets (NRC, 2005).

It is reasonable to assume that these background sources will provide potassium and phosphorus as significantly higher levels in the diet of poultry and cattle than will be carried over from the use as a fermentation aid in the production of microbial strains by ASCUS.

3.3 Metabolic Fate

On ingestion by animals, monopotassium phosphate will dissociate to the respective potassium, hydrogen and phosphate ions. Equivalent behavior in the gastrointestinal tract is observed on ingestion

of related salts such as mono- and di-sodium phosphate and dipotassium phosphate. Thus, the use of monopotassium phosphate will result in exposure by animals to ions commonly consumed in animal feed. On this basis, the available safety data on sodium, calcium and ammonium phosphate salts as well as dipotassium phosphate may be extrapolated to support the safety of monopotassium phosphate (see Section 3.3 and 3.4).

3.4 Mineral Tolerances

Both potassium and phosphorus are required nutrients for poultry and cattle and are considered by the National Research Council (NRC) to be of medium concern for animal health. The NRC has set maximum tolerable levels for potassium of 1% in the diet of poultry and cattle on a dry matter basis, and for phosphorus of 1% for growing birds, 0.8% for laying hens and 0.7% for cattle on a dry matter basis (NRC, 2005). Any carry-over in the diet of monopotassium phosphate from the production of microbial strains for use as DFM products will contribute to the levels of these minerals in the feed but the overall impact on the daily intakes by animals is expected to be low.

3.5 Evaluations by Scientific Bodies

3.5.1 JECFA Evaluation

The Joint FAO/WHO Committee on Food Additives (JECFA) has evaluated the safety of phosphoric acid and phosphate salts as a group, including within the scope of the review, mono-, di- and tri-potassium phosphate (JECFA, 1982). In the latest evaluation conducted in 1982, JECFA concluded that:

"*Metabolically, the phosphate salts provide a source of the various cations and phosphate ion. Of the greatest concern is the toxicity arising from calcium, magnesium and phosphate imbalance in the diet. Phosphate salts were not mutagenic in a number of test systems. Teratogenic effects have not been observed in mammalian test systems.*

Numerous animal studies have shown that excessive dietary phosphorus causes an increase of plasma phosphorus and a decrease in serum calcium. The resulting hypocalcemia stimulates excretion of PTH which in turn increases the rate of bone resorption and decreases calcium excretion. These homeostatic adjustment to high dietary phosphorus may result in bone loss and calcification of soft tissues in animals.

The dose levels of phosphate producing nephrocalcinosis were not consistent among the various rat feeding studies. However, the rat is exquisitely susceptible to calcification and hydronephrosis upon exposure to acids forming calcium chelates or complexes. The lowest dose levels that produce nephrocalcinosis overlap the higher dose levels failing to do so. However, this may be related to other dietary imbalances, such as the level of magnesium in the diet. There is still uncertainty on the optimal Ca:P ratio and whether this ratio is of any dietary significance in man.

The lowest level of phosphate that produced nephrocalcinosis in the rat (1% P in the diet) is used as the basis for the evaluation and, by extrapolation based on the daily food intake of 2800 calories, this give a dose level of 6600 mg P per day as the best estimate of the lowest level that might conceivably cause nephrocalcinosis in man. The usual calculation for provision of a margin of safety is probably not suitable for food additives which are also nutrients. Ingested phosphates from natural sources should be considered together with that from food additive sources. Since phosphorus (as phosphates) is an

essential nutrient and an unavoidable constituent of food, it is not feasible or appropriate to give a range of values from zero to maximum."

On the basis of the above, the maximum tolerable daily intake for man was estimated to be 70 mg/kg body weight.

3.5.2 SCF Evaluation

The Scientific Committee on Food (SCF) in the European Union (EU) evaluated the group of phosphate salts used as food additives in 1990 and agreed with the JECFA estimate of 70 mg/kg body weight for man, calculated as phosphorus (SCF, 1990).

3.5.3 Summary

Taken together the body of available data indicate that the safety of monopotassium phosphate can be considered from the available data on phosphoric acid and phosphate, which have been previously evaluated by JECFA and the SCF for use as food additives. These evaluations highlighted the role of phosphate salts to provide a metabolic source of cations and the phosphate ion. Safety was primarily based on the absence of any genotoxicity and the requirement to provide nutritionally balanced levels in the diet which do not exceed the maximum that can be tolerated by the body.

4. SUMMARY AND CONCLUSIONS

Monopotassium phosphate has an established history of safe use as a mineral substance for use in animal feed in Canada and in the EU. On ingestion by poultry or cattle, monopotassium phosphate will dissociated into the potassium, hydrogen and phosphate ions. For this reason, and consistent with the evaluations of the additive for use in food by JECFA and the SCF, the safety can be primarily derived from the body of available data on phosphoric acid and phosphate salts. Potassium and phosphate are both essential nutrients for animals and present naturally in the feed as well as being added in the form of supplemental salts. The carry-over of potassium and phosphate from its use as a salt in the fermentation of microbial strains for use as DFMs in poultry and cattle feed are not expected to make any significant contribution to the levels present in the diet from natural and supplemental sources. Together, it may be concluded that there are no safety concerns associated with the use of monopotassium phosphate by ASCUS as a fermentation aid under the conditions of intended use.

5. REFERENCES

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Para-Aminobenzoic Acid

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Specification for Aminobenzoic Acid, USP (AM150)

Item Number	AM150
Item	Aminobenzoic Acid, USP
CAS Number	150-13-0
Molecular Formula	$C_7H_7NO_2$
Molecular Weight	137.14
MDL Number	
Synonyms	p-Aminobenzoic Acid ; PABA

Test	Specification	
	Min	Max
ASSAY (DRIED BASIS)	98.0	102.0 %
LOSS ON DRYING		0.2 %
RESIDUE ON IGNITION		0.1 %
ELEMENTAL IMPURITIES:		
LEAD (Pb)	AS REPORTED	
ARSENIC (As)	AS REPORTED	
ANILINE		10 ppm
p-TOLUIDINE		10 ppm
ORGANIC IMPURITIES:		
4-NITROBENZOIC ACID		0.2 %
BENZOCAINE		0.2 %
ANY INDIVIDUAL, UNSPECIFIED IMPURITY		0.1 %
TOTAL IMPURITIES		0.5 %
IDENTIFICATION A) FTIR	SPECTRUM MATCHES REFERENCE	
IDENTIFICATION B (HPLC)	RETENTION TIME MATCHES STANDARD	
EXPIRATION DATE		
DATE OF MANUFACTURE		
APPEARANCE		
RESIDUAL SOLVENTS		AS REPORTED

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(b) (4)

Yeast Extract

(b) (4)

DESCRIPTION

(b) (4) s a primary yeast extract obtained by the autolysis of a selected strain of ***Saccharomyces cerevisiae*** **yeast, especially grown on a molasses based media.**

CERTIFICATION

This product is guaranteed to be Non-GM, free of animal origin ingredient and Kosher.

APPLICATIONS

Recommended for most fermentation processes and laboratory media formulations :
High quality source of readily available soluble, amino acids, peptides, vitamins and essential elements.

PHYSICO-CHEMICAL CHARACTERISTICS

Solubility Totally soluble
Colour of commercial product Light beige
Colour in 10 % solution Light clear yellow

Composition
in g per 100 g of product as is

Dry matter	**94.0**	**-**	**98.0**
Total nitrogen	**10.0**	**-**	**11.8**
Amino nitrogen	**4.5**	**-**	**5.8**
pH	**6.8**	**-**	**7.2**
Sodium chloride		**<**	**0.5**
Proteins (Nitrogen x 6.25)	62.5	-	73.8
Total carbohydrates	7.0	-	13.0
Ash	11.5	-	16.0

MICROBIOLOGICAL CHARACTERISTICS

CFU per g of product

Total plate count	**<**	**5 000**
Coliforms	**<**	**5**
Spores of Clostridium perfringens	**<**	**10**
Yeasts	**<**	**50**
Moulds	**<**	**50**
Salmonella *(per 25 g)*		**Negative**
E.coli		**Negative**
Staphylococcus aureus		**Negative**

(Guaranteed values are listed in bold ; other values are given for indication only)

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AVERAGE AMINO ACID COMPOSITION

expressed in 100 g of raw proteins

Amino acid	Value	Amino acid	Value
Alanine	8.8	Lysine	8.0
Arginine	5.1	Methionine	1.4
Aspartic acid	9.9	Phenylalanine	3.7
Cystine	0.9	Proline	4.0
Glutamic acid	16.3	Serine	4.6
Glycine	4.8	Threonine	4.6
Histidine	2.1	Tyrosine	2.4
Isoleucine	5.5	Tryptophan	1.3
Leucine	7.6	Valine	5.9

AVERAGE VITAMIN COMPOSITION

in mg per kg (ppm) (dry matter)

Vitamin	Min		Max
B1 (Thiamine)	15	-	110
B2 (Riboflavin)	80	-	130
B5 (Calcium Pantothenate)	200	-	400
B6 (Pyridoxine)	30	-	100
B8 (Biotin)	3	-	10
B9 (folic acid)	15	-	60
B12 (Cyanocobalamine) *(µg/kg)*	1	-	5
PP (Niacin)	600	-	1000

MINERALS

in g per 100 g of product as is

Mineral	Min		Max
Sodium		<	0.5
Potassium	4.5	-	6.3
Phosphorus	1.0	-	2.7
Calcium *(ppm)*	100	-	300
Magnesium *(ppm)*	150	-	800
Selenium *(ppm)*		<	0.1
Zinc *(ppm)*		<	90

HEAVY METALS

in mg per kg (ppm)

Metal	Value
Arsenic	< 0.5
Cadmium	< 0.1
Mercury	< 0.05
Lead	< 0.2

PACKAGING

Powder :
- 25 kg sealed paper bags with polyethylene liner / pallets of 750 or 1000 kg,
- 25 kg carboard boxes with polyethylene liner / pallets of 900 kg,
- 500 kg big bags.

Microgranulated powder :
- 25 kg sealed paper bags with polyethylene liner / pallets of 500 or 600 kg,
- 25 kg carboard boxes with polyethylene liner / pallets of 720 kg,
- 500 or 700 kg big bags.

SHELF LIFE & STORAGE

3 years in their original packaging, stored in a cool and dry place protected from direct sun-light.

The information contained in this data sheet is accurate to the best of our knowledge at the indicated date and remains our property.
It is the user's responsibility to ensure that the conditions and possible uses of the product conform in particular to current laws and regulations.

0.75 mg/mL to another conical flask. Add 1 mL of *Fehling's Solution A* and of *Fehling's Solution B* (see *Cupric Tartrate TS, Alkaline*, under *Solutions and Indicators*) to each flask, heat to boiling, and cool. The sample solution is less turbid than the dextrose solution, which forms a red-brown precipitate.
Residue on Ignition Determine as directed under *Residue on Ignition*, Appendix IIC, igniting a 2-g sample.
Water Determine as directed under *Water Determination*, Appendix IIB.

Packaging and Storage Store in well-closed containers in a dry place.

Yeast, Autolyzed

Autolyzed Yeast

DESCRIPTION

Yeast, Autolyzed, occurs in granular, powdered, flake, or paste form. It is the concentrated, nonextracted, partially soluble digest obtained from food-grade yeasts. Solubilization is accomplished by enzyme hydrolysis or autolysis of yeast cells. Food-grade salts and enzymes may be added. Yeast, Autolyzed contains both soluble and insoluble components derived from the whole yeast cell. It is composed primarily of amino acids, peptides, proteins, carbohydrates, fats, and salts.

Function Flavoring agent; flavor enhancer; protein source; binder.

REQUIREMENTS

Note: Perform all analyses after drying. Liquid and paste samples should be evaporated to dryness on a steam bath, then, as for the powdered and granular forms, dried to constant weight at 65° (see *General Provisions*).

Assay Not less than 6.1% total nitrogen, which is equivalent to not less than 38.1% protein (%N × 6.25), calculated on the sodium chloride-free basis.
α-Amino Nitrogen/Total Nitrogen (AN/TN) Percent Ratio Not less than 5.0%.
Ammonia Nitrogen Not more than 1.0%, calculated on the sodium chloride-free basis.
Glutamic Acid Not more than 13.0% of glutamic acid ($C_4H_7NO_4$), calculated on the sodium chloride-free basis, and not more than 24.0% of the total amino acids.
Insoluble Matter Between 20.0% and 60.0%.
Lead Not more than 2 mg/kg.
Mercury Not more than 3 mg/kg.
Microbial Limits:
Aerobic Plate Count Not more than 50,000 CFU per gram.
Coliforms Not more than 10 CFU per gram.
Salmonella Negative in 25 g.
Yeasts and Molds Not more than 50 CFU per gram.
Potassium Not more than 13.0%.
Sodium Chloride Not more than 43.0%.

TESTS

Assay Determine as directed under *Nitrogen Determination*, Appendix IIIC. Calculate the percent protein (P) by the formula

$$P = 6.25N,$$

in which N is the percent nitrogen.
α-Amino Nitrogen/Total Nitrogen (AN/TN) Percent Ratio Determine *α-Amino Nitrogen* as directed under *α-Amino Nitrogen Determination*, Appendix IIIC. Determine *Total Nitrogen* as directed under *Nitrogen Determination*, Appendix IIIC. Calculate the AN/TN percent ratio by dividing the percent α-amino nitrogen (AN) by the percent total nitrogen (TN) as corrected for ammonia nitrogen (NH_3-N) according to the formula

$$100[(AN - NH_3\text{-}N)/(TN - NH_3\text{-}N)].$$

Ammonia Nitrogen Determine as directed under *Ammonia Nitrogen*, Appendix IIIC.
Glutamic Acid Determine as directed under *Glutamic Acid*, Appendix IIIC.
Insoluble Matter Transfer about 5 g of sample, accurately weighed, into a 250-mL Erlenmeyer flask. Add 75 mL of water, cover the flask with a watch glass, and boil gently for 2 min. Filter the solution through a tared filtering crucible, dry at 105° for 1 h, cool, and weigh.
Lead Determine as directed in the *Flame Atomic Absorption Spectrophotometric Method* under *Lead Limit Test*, Appendix IIIB, using a 10-g sample.
Mercury Determine as directed under *Mercury Limit Test*, Appendix IIIB.
Microbial Limits (**Note:** Current methods for the following tests may be found online at <www.cfsan.fda.gov/~ebam/bam-toc.html>):
Aerobic Plate Count
Coliforms
Salmonella
Yeasts and Molds
Potassium
Spectrophotometer Use any suitable atomic absorption spectrophotometer.
Standard Solution Transfer 38.20 mg of reagent-grade potassium chloride, accurately weighed, into a 100-mL volumetric flask, dissolve in and dilute to volume with deionized water, and mix. Transfer 5.0 mL of this solution to a 1000-mL volumetric flask, dilute to volume with deionized water, and mix. Each milliliter contains 1.0 µg of potassium (K).

Sample Solution Transfer 2.33 g of a previously dried sample, accurately weighed, into a silica or porcelain dish. Ash in a muffle furnace at 550° for 2 to 4 h. Allow the ash to cool, and dissolve it in 5 mL of 20% hydrochloric acid, warming the solution if necessary to complete solution of the residue. Filter the solution through acid-washed filter paper into a 1000-mL volumetric flask. Wash the filter paper with hot water, dilute the solution to volume, and mix. Prepare a 1:300 dilution of this solution in water to obtain the final *Sample Solution*.

Procedure Determine the absorbance of each solution at 766.5 nm, following the manufacturer's instructions for optimum operation of the spectrophotometer. The absorbance of the *Sample Solution* does not exceed that of the *Standard Solution*.

Sodium Chloride

Spectrophotometer Use any suitable atomic absorption spectrophotometer.

Standard Solution Transfer 43.0 mg of reagent-grade sodium chloride, accurately weighed, into a 100-mL volumetric flask, dissolve in and dilute to volume with deionized water, and mix. Using water as the solvent, prepare a 1:100 dilution of this solution to obtain the final working *Standard Solution*. Each milliliter contains 4.3 µg of sodium chloride (NaCl).

Sample Solution Transfer 1.00 ± 0.05 g of a previously dried sample, accurately weighed, into a silica or porcelain dish. Ash in a muffle furnace at 550° for 2 to 4 h. Allow the ash to cool, and dissolve it in 5 mL of 20% hydrochloric acid, warming the solution if necessary to complete solution of the residue. Filter the solution through acid-washed filter paper into a 100-mL volumetric flask. Wash the filter paper with hot water, dilute the solution to volume, and mix. Using water as the solvent, prepare a 1:100 dilution of this solution to obtain the final *Sample Solution*.

Procedure Determine the absorbance of each solution at 589.0 nm, following the manufacturer's instructions for optimum operation of the spectrophotometer. The absorbance produced by the *Sample Solution* does not exceed that of the *Standard Solution*.

Packaging and Storage Store in well-closed containers.

Yeast, Dried

Brewer's Yeast; Dried Yeast; Torula Yeast

DESCRIPTION

Yeast, Dried, occurs as a light brown to buff powder, granules, or flakes. It is the comminuted, washed, dried, and pasteurized cell walls from *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, or *Torula utilis*. It contains no added substances.

Function Carrier; flavor enhancer.

REQUIREMENTS

Identification When examined under a microscope, a sample exhibits numerous irregular masses and isolated yeast cells—the latter ovate, elliptical, spheroidal, or elliptic-elongate in shape, some with one or more attached buds—up to 12 µm in length and up to 7.5 µm in width. Each has a wall of cellulose surrounding a protoplast containing refractile glycogen vacuoles and oil globules.

Assay Not less than 45.0% protein.

Ash (Total) Not more than 8.0%.

Folic Acid Not more than 0.04 mg/g.

Lead Not more than 1 mg/kg.

Loss on Drying Not more than 7.0%.

Microbial Limits:

Aerobic Plate Count Not more than 7500 CFU per gram.

Coliforms Not more than 10 CFU per gram.

Salmonella Negative in 25 g.

TESTS

Assay Determine the percent nitrogen as directed under *Nitrogen Determination*, Appendix IIIC, and multiply by 6.25 to obtain the percent protein.

Ash (Total) Determine as directed under *Ash (Total)*, Appendix IIC.

Folic Acid (**Note**: In the microbiological assay of folic acid, the microorganism is highly sensitive to minute amounts of growth factors and to many cleansing agents. Meticulously cleanse 20- × 150-mm test tubes and other necessary glassware with a suitable detergent, sodium lauryl sulfate, or an equivalent substitute. Follow cleansing by heating for 1 to 2 h at approximately 250°.) This method is based on AOAC method 960.46.

Vitamin-Free, Acid-Hydrolyzed Casein Solution Prepare the solution by mixing 400 g of vitamin-free casein with 2 L of boiling 5 *N* hydrochloric acid. Autoclave for 10 h at 121°. Concentrate the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the paste in water, adjust the solution to pH 3.5 ± 0.1 with a 10% solution of sodium hydroxide, and dilute with water to a final volume of 4 L. Add 80 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Filter the solution if a precipitate forms on storage.

Adenine–Guanine–Uracil Solution Dissolve 1.0 g each of adenine sulfate, guanine hydrochloride, and uracil in 50 mL of warm 1:2 hydrochloric acid, cool, and dilute with water to 1 L.

Asparagine Solution Dissolve 10 g of L-asparagine monohydrate in approximately 500 mL of water, and dilute with water to 1 L.

Manganese Sulfate Solution Dissolve 2.0 g of manganese sulfate monohydrate in water, and dilute with water to 200 mL.

Polysorbate 80 Solution Dissolve 25 g of polysorbate 80 (polyoxyethylene sorbitan monooleate) in ethyl alcohol, and dilute with ethyl alcohol to make 250 mL.

Salt Solution Dissolve 20 g of magnesium sulfate heptahydrate, 1 g of sodium chloride, 1 g of ferrous sulfate heptahydrate, and 1 g of manganese sulfate monohydrate in water, dilute with water to 1 L, add 10 drops of hydrochloric acid, and mix.

Tryptophan Solution Suspend 2.0 g of L-tryptophan in 800 mL of water, heat to 80°, and add, dropwise and while stirring, 1:2 hydrochloric acid until the suspension dissolves. Cool, and dilute with water to 1 L.

Vitamin Solution Dissolve 10 mg of *p*-aminobenzoic acid, 8 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 4 mg of thiamine hydrochloride, 8 mg of niacin, and 0.2 mg of biotin in approximately 300 mL of water. Add 10 mg of riboflavin dissolved in approximately 200 mL of 0.02 *N* acetic acid. Add a solution containing 1.9 g of anhydrous sodium acetate and 1.6 mL of glacial acetic acid in approximately 40 mL of water. Dilute the solution with water to a final volume of 2 L.

Xanthine Solution Suspend 1.0 g of xanthine in 200 mL of water, heat to approximately 70°, add 30 mL of 2:5 ammonium hydroxide, and stir until the suspension dissolves. Cool, and dilute with water to 1 L.

Basal Medium Stock Solution Prepare the solution by adding, with mixing, in the following order, 25 mL of the *Vitamin-Free, Acid-Hydrolyzed Casein Solution*, 25 mL of the *Tryptophan Solution*, 2.5 mL of the *Adenine–Guanine–Uracil Solution*, 5 mL of the *Xanthine Solution*, 15 mL of the *Asparagine Solution*, 50 mL of the *Vitamin Solution*, and 5 mL of the *Salt Solution*. Add approximately 50 mL of water, and add, with mixing, 0.19 g of L-cysteine monohydrochloride monohydrate, 10 g of anhydrous glucose, 13 g of sodium citrate dihydrate, 1.6 g of anhydrous dipotassium hydrogen phosphate, and 0.0013 g of glutathione. When solution is complete, adjust to pH 6.8 with 10% sodium hydroxide solution, and add, with mixing, 0.25 mL of the *Polysorbate 80 Solution* and 5 mL of the *Manganese Sulfate Solution*. Dilute to a final volume of 250 mL with water.

Liquid Culture Medium Dissolve 15 g of peptonized milk, 5 g of water-soluble yeast extract, 10 g of anhydrous glucose, and 2 g of anhydrous potassium dihydrogen phosphate in about 600 mL of water. Add 100 mL of filtered tomato juice (filtered through Whatman No. 1 filter paper, or equivalent), and adjust to pH 6.5 by the dropwise addition of 1.0 *N* sodium hydroxide. Add, with mixing, 10 mL of the *Polysorbate 80 Solution*. Dilute with water to a final volume of 1000 mL. Add 10-mL portions of this *Liquid Culture Medium* to test tubes, cover to prevent contamination, and sterilize by heating in an autoclave at 121° for 15 min. Cool the tubes rapidly to keep color formation to a minimum, and store at 10° in the dark.

Agar Culture Medium Add 6.0 g of agar to 500 mL of *Liquid Culture Medium*, and heat with stirring on a steam bath until the agar dissolves. Add approximately 10-mL portions of the hot solution to test tubes, cover to prevent contamination, sterilize by heating in an autoclave at 121° for 15 min, and cool tubes in an upright position to keep color formation to a minimum. Store at 10° in the dark.

Suspension Medium Dilute an appropriate volume of the *Basal Medium Stock Solution* with an equal volume of water. Distribute 10-mL portions of this *Suspension Medium* to test tubes, cover to prevent contamination, sterilize by heating in an autoclave at 121° for 15 min, and cool tubes rapidly to keep color formation to a minimum. Store at 10° in the dark.

Assay Organism Maintain *Enterococcus* (*Streptococcus*) *faecalis* ATCC 8043 by subculturing in stab cultures of *Agar Culture Medium* and incubating at 37° for 24 h. Stab cultures may be stored in the dark at 10° for a maximum of 7 days until use. Prepare fresh stab cultures at least on a weekly basis. Before using a new culture in the assay, make several successive transfers of the culture over a 1- to 2-week period. Transfer cells from the stab culture of *Assay Organism* to a sterile tube containing 10 mL of *Liquid Culture Medium*. Incubate for 18 h at 37°. Under aseptic conditions, centrifuge the culture, and decant the supernate. Wash the cells with three 10-mL portions of sterile *Suspension Medium*. Resuspend cells in 10 mL of sterile *Suspension Medium*—these cells serve as the inoculum.

Folic Acid Stock Solutions Accurately weigh, in a closed system, 50 to 60 mg of USP Folic Acid Reference Standard that has been dried to constant weight and stored in the dark over phosphorus pentoxide in a desiccator. Dissolve in approximately 30 mL of 0.01 *N* sodium hydroxide, add approximately 300 mL of water, adjust to pH 7.5 with 1:2 hydrochloric acid, and dilute with additional water to a final folic acid concentration of exactly 100 µg/mL. Store under toluene in the dark at 10°.

Prepare an intermediate *Folic Acid Stock Solution* containing 1 µg/mL by placing 10 mL of the 100 µg/mL *Folic Acid Stock Solution* in a flask, adding approximately 500 mL of water, adjusting to pH 7.5 with dilute hydrochloric acid or sodium hydroxide as necessary, and diluting with additional water to a final volume of 1 L. Store under toluene in the dark at 10°.

Prepare the final *Folic Acid Stock Solution* by taking 100 mL of the intermediate *Folic Acid Stock Solution*, adding approximately 500 mL of water, adjusting to pH 7.5 with dilute hydrochloric acid or sodium hydroxide as necessary, and diluting with additional water to a final volume of 1 L. Store under toluene in the dark at 10°. This final *Folic Acid Stock Solution* has a concentration of 100 ng/mL.

Preparation of the Standard Curve Dilute the *Folic Acid Stock Solution* with water to a measured volume such that after incubation, as described below, response at the 5.0-mL level of this solution is equivalent to a titration volume of 8 to 12 mL. This concentration is usually 1 to 4 ng of folic acid per mL but can vary with the culture used in the assay. Designate this solution as the *Folic Acid Working Standard Solution*. To duplicate test tubes, add 0.0 (for uninoculated blanks), 0.0 (for inoculated blanks), 1.0, 2.0, 3.0, 4.0, and 5.0 mL, respectively, of the *Folic Acid Working Standard Solution*. Add water to each tube to make a final volume of 5.0 mL. Add 5.0 mL of the *Basal Medium Stock Solution* to each tube, and mix. Cover the tubes suitably to prevent bacterial contamination, and sterilize by heating in an autoclave at 121° for 10 min. Cool tubes rapidly to keep color formation to a minimum.

Note: Sterilizing and cooling conditions must be kept uniform to obtain reproducible results.

Aseptically inoculate each tube with 1 drop of the *Assay Organism* inoculum, except for one set of duplicate tubes containing 0.0 mL of the *Folic Acid Working Standard Solution*, which serve as the uninoculated blanks. Incubate the tubes for 72 h at 37°.

Note: Contamination of assay tubes with any foreign organism invalidates the assay.

Titrate the contents of each tube with 0.1 *N* sodium hydroxide, using bromothymol blue as the indicator. Disregard the results of the assay if the titration volume for the inoculated blank is more than 1.5 mL greater than that for the uninoculated blank. The titration volume for the 5.0-mL level of the *Folic Acid Working Standard Solution* should be approximately 8 to 12 mL. Prepare a standard curve by plotting the titration values, expressed in milliliters of 0.1 *N* sodium hydroxide for each level of the *Folic Acid Working Standard Solution* used, against the amount of folic acid contained in that tube.

Assay Solution Weigh and suspend 1.0 g of sample in 100 mL of water. Add 2 mL of 2:5 ammonium hydroxide. If the sample is not readily soluble, comminute to disperse it evenly in the liquid, then agitate vigorously and wash down the sides of the flask with 0.1 *N* ammonium hydroxide. Heat the mixture in an autoclave at 121° for 15 min. If lumping occurs, agitate the sample until the particles are evenly dispersed. Dilute the mixture with water to 200 mL. Filter through Whatman No. 1 filter paper, or equivalent, if necessary, to remove any undissolved particles. Adjust the filtered mixture to pH 6.8 and dilute to 1000 mL with water. Prepare the final *Assay Solution* by diluting 1.0 mL of the intermediate solution with water to a final volume of 50.0 mL.

Procedure To duplicate test tubes, add 0.0 (for uninoculated blanks), 0.0 (for inoculated blanks), 1.0, 2.0, 3.0, 4.0, and 5.0 mL, respectively, of the *Assay Solution*. Add water to each tube to make a final volume of 5.0 mL. Proceed as directed above for *Preparation of the Standard Curve*. Determine the amount of folic acid for each level of the *Assay Solution* by interpolation from the standard curve. Discard any observed titration values equivalent to less than 0.5 mL or more than 4.5 mL of the *Folic Acid Working Standard Solution*. If necessary, the *Assay Solution* can be diluted to achieve the ideal concentration range of folic acid. For each level of *Assay Solution* used, calculate the vitamin content per milliliter of *Assay Solution*. Calculate the average vitamin content of values obtained from tubes that do not vary by greater than 10% from this average. More than two-thirds of the original number of tubes must be within 10% of the average folic acid value, or the data cannot be used to calculate the folic acid concentration in the sample. If the data are acceptable, determine the folic acid concentration in the sample by multiplying the average folic acid concentration, in nanograms per milliliter, of the *Assay Solution* by 0.025 to give the milligrams of folic acid per gram of sample.

Lead Determine as directed for *Method II* in the *Atomic Absorption Spectrophotometric Graphite Furnace Method* under *Lead Limit Test*, Appendix IIIB.

Loss on Drying Determine as directed under *Loss on Drying*, Appendix IIC, drying a 1-g sample at 105° for 4 h.

Microbial Limits (**Note:** Current methods for the following tests may be found online at <www.cfsan.fda.gov/~ebam/bam-toc.html>):

Aerobic Plate Count
Coliforms
Salmonella

Packaging and Storage Store in tight containers in a cool, dry place.

Yeast Extract

Autolyzed Yeast Extract

DESCRIPTION

Yeast Extract occurs as a liquid, paste, powder, or granular substance. It comprises the water-soluble components of the yeast cell, the composition of which is primarily amino acids, peptides, carbohydrates, and salts. Yeast Extract is produced through the hydrolysis of peptide bonds by the naturally occurring enzymes present in edible yeasts or by the addition of food-grade enzymes. Food-grade salts may be added during processing.

Function Flavoring agent; flavor enhancer.

REQUIREMENTS

Note: Perform all calculations on the dried basis. In a suitable tared container, evaporate liquid and paste samples to dryness on a steam bath, then, as for the powdered and granular forms, dry to constant weight at 105° (see *General Provisions*).

Assay (Protein) Not less than 42.0% protein.
α-Amino Nitrogen/Total Nitrogen (AN/TN) Percent Ratio Not less than 15.0% or more than 55.0%.
Ammonia Nitrogen Not more than 2.0%, calculated on a dry, sodium chloride-free basis.
Glutamic Acid Not more than 12.0% as $C_5H_9NO_4$ and not more than 28.0% of the total amino acids.
Insoluble Matter Not more than 2%.
Lead Not more than 2 mg/kg.
Mercury Not more than 3 mg/kg.
Microbial Limits:
Aerobic Plate Count Not more than 50,000 CFU per gram.
Coliforms Not more than 10 CFU per gram.
Salmonella Negative in 25 g.
Yeasts and Molds Not more than 50 CFU per gram.
Potassium Not more than 13.0%.
Sodium Chloride Not more than 50.0%.

TESTS

Assay (Protein) Determine as directed under *Nitrogen Determination*, Appendix IIIC. Calculate the percent protein (*P*) by the equation

$$P = 6.25N,$$

in which *N* is the percent nitrogen.

α-Amino Nitrogen/Total Nitrogen (AN/TN) Percent Ratio Determine *α-Amino Nitrogen* as directed under *α-Amino Nitrogen Determination*, Appendix IIIC. Determine *Total Nitrogen* as directed under *Nitrogen Determination*, Appendix IIIC. Calculate the *AN/TN* percent ratio, in which *AN* is the percent of α-amino nitrogen and *TN* is the percent of total nitrogen.

Ammonia Nitrogen Determine as directed under *Ammonia Nitrogen*, Appendix IIIC.

Glutamic Acid Determine as directed under *Glutamic Acid*, Appendix IIIC.

Insoluble Matter Transfer about 5 g of sample, accurately weighed, into a 250-mL Erlenmeyer flask, add 75 mL of water, cover the flask with a watch glass, and boil gently for 2 min. Filter the solution through a tared filtering crucible, dry at 105° for 1 h, cool, and weigh.

Lead Determine as directed in the *Flame Atomic Absorption Spectrophotometric Method* under *Lead Limit Test*, Appendix IIIB, using a 10-g sample.

Mercury Determine as directed under *Mercury Limit Test*, Appendix IIIB.

Microbial Limits (**Note:** Current methods for the following tests may be found online at <www.cfsan.fda.gov/~ebam/bam-toc.html>):

Aerobic Plate Count
Coliforms
Salmonella
Yeasts and Molds

Potassium Proceed as directed in the monograph for *Yeast, Autolyzed*.

Sodium Chloride Proceed as directed in the monograph for *Yeast, Autolyzed*, except to use 50.0 mg of reagent-grade sodium chloride to prepare the *Standard Solution*.

Packaging and Storage Store in well-closed containers.

Zein

CAS: [9010-66-6]

DESCRIPTION

Zein occurs as a very light yellow to tan colored, granular or fine powder. It comprises the prolamine protein component of corn (*Zea mays* Linne'). It is produced commercially by extraction from corn gluten with alkaline aqueous isopropyl alcohol. The extract is then cooled, which causes the Zein to precipitate. It is insoluble in water.

Function Surface-finishing agent; texturizing agent.

REQUIREMENTS

Identification

A. Dissolve about 0.1 g of sample in 10 mL of 0.1 *N* sodium hydroxide, and add a few drops of cupric sulfate TS. Warm in a water bath. A purple color appears.

B. Add 1 mL of nitric acid to a test tube containing 25 mg of sample. Agitate vigorously. The solution turns light yellow. Further addition of about 10 mL of 6 *N* ammonium hydroxide produces an orange color.

Assay Not less than 88.0% and not more than 96.0% protein, calculated on the dried basis.

Lead Not more than 2 mg/kg.

Loss on Drying Not more than 8.0%.

Loss on Ignition Not more than 2%.

TESTS

Assay Determine as directed under *Nitrogen Determination*, Appendix IIIC. Calculate the percent protein (*P*) by the equation

$$P = 6.25N,$$

in which *N* is the percent nitrogen.

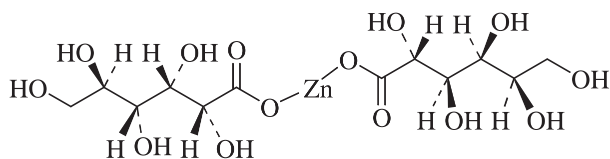
Lead Determine as directed in the *Flame Atomic Absorption Spectrophotometric Method* under *Lead Limit Test*, Appendix IIIB, using a 10-g sample.

Loss on Drying Determine as directed under *Loss on Drying*, Appendix IIC, drying a 2-g sample in an air oven at 105° for 2 h.

Loss on Ignition Determine as directed under *Ash (Total)*, Appendix IIC, using a 2-g sample.

Packaging and Storage Store in well-closed containers.

Zinc Gluconate



$C_{12}H_{22}O_{14}Zn$ Formula wt 455.68

CAS: [4468-02-4]

DESCRIPTION

Zinc Gluconate occurs as a white or nearly white, granular or crystalline powder and as a mixture of various states of

ASCUS
BIOSCIENCES

Specifications for Salt

Ingredient:	Sodium Chloride
Chemical Nomenclature:	NaCl
Specifications:	Feed/Food Grade or FCC
Moisture:	≤ 1.5% by LOD
Purity:	≥ 95%

Sodium Hydroxide 1M

(b) (4)

50% CAUSTIC SODA
MEMBRANE GRADE

SALES SPECIFICATION

PROPERTY	SPECIFICATION	BASIS	TEST METHOD
SODIUM HYDROXIDE (NAOH)	49.5 - 51.5	WT. %	TITRIMETRY
SODIUM OXIDE (NA_2O)	38.3 – 40.0	WT. %	CALCULATED
SODIUM CARBONATE (NA_2CO_3)	.050 MAX.	WT. %.	TITRIMETRY
SODIUM CHLORIDE (NACI)	.0075 MAX.	WT. %	TURBIDIMETRY
SODIUM CHLORATE ($NACIO_3$)	10.0 MAX.	PPM	ION CHROMOTAGRAPHY
SODIUM SULFATE (NA_2SO_4)	30 MAX.	PPM	ION CHROMOTAGRAPHY
IRON (FE)	2.0 MAX.	PPM	COLORIMETRY

ALL RESULTS ARE ON A SOLUTION BASIS.

Sodium Iodide

(b) (4)

Specification for Sodium Iodide, USP (SO175)

Item Number	SO175
Item	Sodium Iodide, USP
CAS Number	7681-82-5
Molecular Formula	NaI
Molecular Weight	149.89
MDL Number	
Synonyms	

Test	Specification	
	Min	Max
ASSAY (NaI)	99.0 - 101.5 %	
ALKALINITY		TO PASS TEST
WATER		2.0 %
IODATE (IO_3)		TO PASS TEST
NITRATE, NITRITE, AND AMMONIA		TO PASS TEST
THIOSULFATE AND BARIUM		TO PASS TEST
HEAVY METALS (as Pb)		0.001 %
POTASSIUM (K)		TO PASS TEST
IDENTIFICATION		TO PASS TEST
RETEST DATE		
RESIDUAL SOLVENTS		TO PASS TEST

(b) (4)

(b) (4) Soy Peptone F

(b) (4)

SOY PEPTONE F – A1603

Description

Origin : Soy Peptone F is obtained from defatted soybean flour, using Identity-Preserved (IP) raw materials as it pertains to Genetically Modified Organisms (GMO). The digestive enzyme papain is not concerned by GMO issues.

Regulatory : this peptone is classified animal free, GMO free (according to the European Directive 2001/18/CE) but can not be considered allergen-free due to the presence of soy proteins (Annex IIIa of the EU directive 2003/89/EC, updated with 2006/142/EC). **Kosher certification is available.**

Application : produced and fortified for principle applications in the fermentation industry according to a proprietary formula, this peptone leads to the rapid and luxuriant growth of many microorganisms, including yeasts and molds.

Physical properties

Appearance : beige powder
Stability (2% solution) : stable
Solubility in water at 2% : total

Microbiological controls

Total aerobic mesophilic flora ≤ 5000 cfu/g

Chemical analysis

Total nitrogen (N_T) : 10.0%
α-amino nitrogen ($N\alpha$) : 2.5%
$N\alpha$ / N_T : 0.25
Sulfuric ash : 13.0%
pH (2% in solution) : 7.0
Total carbohydrates : approx. 15.0 - 20.0%
Chlorides (as NaCl) : 2.0%
Loss on drying ≤ 6.0%

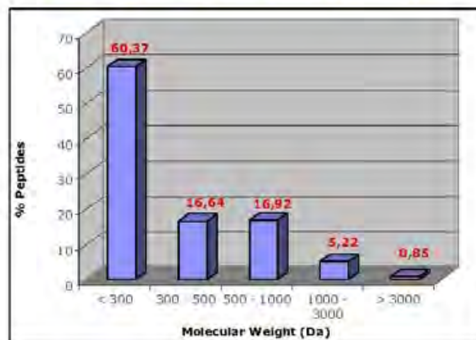
Chemical characteristics

Nitrates : negative
Indole : negative
Aflatoxins (B1, B2, G1, G2) < 2 µg / kg

Amino acid distribution (mg/g)[1]

	Total amino acids		Total amino acids
Aspartic acid	73.0	Methionine	5.0
Threonine	23.0	Isoleucine	25.0
Serine	32.0	Leucine	45.0
Glutamic acid	119.0	Tyrosine	13.0
Proline	30.0	Phenylalanine	30.0
Glycine	28.0	Histidine	17.0
Alanine	29.0	Lysine	39.0
Cysteine	0.00	Arginine	45.0
Valine	37.0	Tryptophan	0.00

Molecular weight distribution (Daltons)



Storage

Keep in original packaging when not in use, in a dry area ideally between 10 and 35°C. Avoid direct sunlight. Hygroscopic product.
Expiry date : 5 years from date of manufacture.

Standard packaging

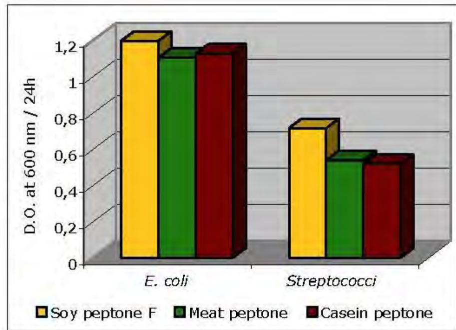
25 kg carton ; other formats inquire.
Delivered with Certificate of Analysis, Certificate of Origin, GMO Attestation for raw materials.

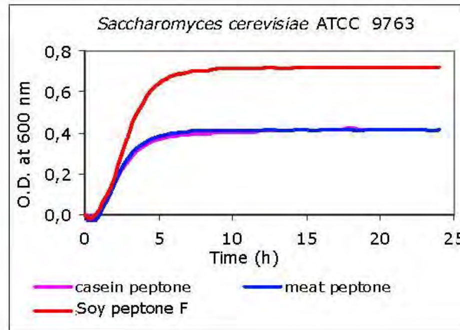
Sanitary Attestation

This plant peptone is classified animal-free by (b) (4) Based on the manufacturing protocol, we attest that no animal raw materials are prescribed for use in the production this product nor are any of the raw materials derived from animal products. Also, to the best of our knowledge, the product contains no genetically modified organisms as defined by current legislation for labelling (absence = less than 0.9%).

OBSERVED MICROBIAL GROWTH POTENTIAL :

Replacement of animal-based substrates : fermentation

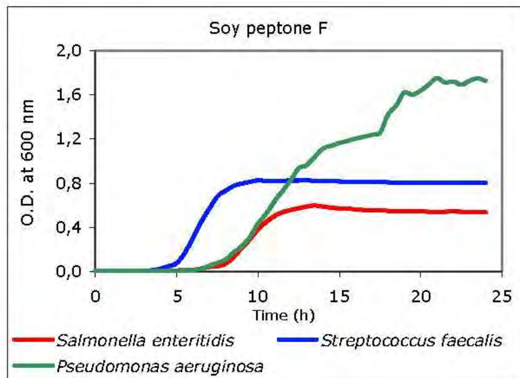




Test conditions :

Inoculum 10^6 cfu / mL
Growth medium: 1 % peptone + 0.5 % NaCl ; pH 7.3

Replacement of animal-based substrates : diagnostic culture media



Test conditions :

Inoculum 10^4 cfu/mL

Culture medium : 3% peptone + 0.25% glucose

pH 7.3

Conclusions :

Laboratory tests demonstrate superior growth as a replacement for bovine substrates or as a stand-alone peptone. Use of Soy peptone F can be recommended in nearly all fermentative applications. Results may differ depending on individual laboratory conditions and for other genera, species and strains.

The information presented in this document is submitted in good faith based on internal testing performed at (b) (4) and represents the best of our knowledge at the present time. It is provided as a guide and no warranty, implied or otherwise is associated with this data, nor is any liability assumed for patent infringement. All data represents typical analyses not to be taken for exact specifications.

End-users are directed to perform proprietary tests to determine suitability and performance for specific applications. The information and results contained in this technical data sheet are susceptible to modification at any time, without warning.

Date of edition : 2008-06-27. Code document : A1603/A/B385-3 : 2

(b) (4)

Thiamine Hydrochloride

(b) (4)

Specification for Thiamine Hydrochloride, FCC (T1053)

Item Number	T1053
Item	Thiamine Hydrochloride, FCC
CAS Number	67-03-8
Molecular Formula	$C_{12}H_{17}ClN_4OS.HCl$
Molecular Weight	337.27
MDL Number	
Synonyms	3-(4-Amino-2-methylpyrimidin-5-ylmethyl)-5-(2-hydroxyethyl)-4-methylthiazolium Chloride

Test	Specification	
	Min	Max
ASSAY ($C_{12}H_{17}ClN_4OS$•HCl)	98.0 - 102.0 %	
COLOR OF SOLUTION		TO PASS TEST
pH OF A 1 IN 100 SOLUTION	2.7 - 3.4	
LEAD (Pb)		2 mg/kg
NITRATE (NO_3)		TO PASS TEST
RESIDUE ON IGNITION		0.2 %
WATER		5.0 %
IDENTIFICATION		TO PASS TEST
EXPIRATION DATE		

(b) (4)

Zinc Chloride

(b) (4)

Specification for Zinc Chloride, Granular, USP (ZI105)

Item Number	ZI105
Item	Zinc Chloride, Granular, USP
CAS Number	7646-85-7
Molecular Formula	$ZnCl_2$
Molecular Weight	136.29
MDL Number	
Synonyms	

Test	Specification	
	Min	Max
ASSAY	97.0	100.5 %
OXYCHLORIDE	TO PASS TEST	
SULFATES (SO_4)		0.03 %
ALKALIES AND ALKALINE EARTHS		1.0 %
AMMONIUM SALTS	TO PASS TEST	
LEAD (Pb)		0.005 %
ELEMENTAL IMPURITIES	AS REPORTED	
IDENTIFICATION A	TO PASS TEST	
IDENTIFICATION B	TO PASS TEST	
CERTIFIED HALAL		
RETEST DATE		
DATE OF MANUFACTURE		
APPEARANCE		
RESIDUAL SOLVENTS	TO PASS TEST	

(b) (4)

Appendix 10 was intentionally left blank