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Identification of fungi for sweet whey permeate utilization and eicosapentaenoic acid production

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SUMMARY

Eighteen selected organisms of the *Eumycota* division of the fungi kingdom were examined for eicosapentaenoic acid production and utilization of sweet whey permeate. The organisms belong to the subdivisions *Mastigomycotina*, *Zygomycotina*, *Ascomycotina* and *Deuteromycotina*. Seven organisms were initially identified as lactose utilizers (the predominant sugar in sweet whey permeate) and eicosapentaenoic acid (EPA) producers. Utilization of lactose was demonstrated and EPA production was confirmed for four organisms, all of the subdivision *Mastigomycotina*. Growth studies showed that *P. ultimum* had the best potential for future work.

INTRODUCTION

Approximately 47% of the whey produced, which amounts to 450 million kg of lactose, is not utilized [4,7]. The majority of whey produced is sweet whey, a by-product of cheese production. Sweet whey permeate (SWP), a concentrated lactose fraction containing approximately 80% lactose (w/w), is produced industrially by separating the lactose fraction from the high molecular weight proteins of sweet whey. SWP is a good medium constituent for organisms which use lactose, since it also contains minerals, proteins and vitamins [4].

The U.S. market for ω-3 fatty acids is estimated to be $100 million [10]. At present, ω-3 fatty acids are marketed in the form of concentrated fish oils. These concentrates are unattractive because they contain low concentrations of ω-3 fatty acids, less desirable fatty acids [8], cholesterol [10], and have the potential of containing high levels of heavy metals and pesticides [10]. The two most important ω-3 fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid which are long chain polyunsaturated fatty acids (PUFAs). EPA has potential pharmaceutical value and may be effective in preventing thrombosis [2,3]. The purpose of this study was to determine if fungi could be used for the production of EPA from lactose.

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MATERIALS AND METHODS

Organisms were examined for lactose, glucose, galactose and SWP utilization, and EPA production using a four-level protocol. First all organisms were examined for lactose and glucose utilization using a dry weight procedure to measure growth on these sugars. Second, those organisms showing lactose utilization in the first level were tested to confirm if they were lactose utilizers by high performance liquid chromatography (HPLC) and tentatively tested for EPA production by gas chromatography (GC). Third, organisms demonstrating lactose utilization and EPA production in the second level were examined for utilization of SWP with a dry weight procedure and confirmation of EPA production using co-chromatography and gas chromatography/mass spectroscopy (GC/MS). Fourth, a growth study was conducted with organisms showing lactose utilization and confirmed EPA production using SWP media. Here lactose, glucose, galactose, dry weight, pH and EPA profiles were measured.

Organisms. Organisms were selected for this study from the *Eumycota* division of the fungi kingdom. The organisms belong to the subdivisions *Mastigomycotina, Zygomycotina, Ascomycotina* and *Deuteromycotina*. The organisms are listed in Table 1 where they are grouped according to their subdivisions.

Maintenance media. Organisms of the genera *Achlya, Dictyuchus, Saprolegnia, Thraustotheca* and *Pythium*, and the organism *C. coronatus* ATCC 24160 were maintained

TABLE 1

Organisms examined for lactose utilization in the first level experiments. Organisms are listed under the subdivisions specified by Ainsworth and Bisby's Dictionary of Fungi [6]

Genus	Organism	Medium
Subdivision: *Mastigomycotina*		
Achlya	*A. bonariensis* ATCC[a] 22407	CM
Achlya	*A. echinulata* ATCC 22408	YM
Dictyuchus	*D. monosporus* ATCC 34931	YM
Saprolegnia	*S. parasitica* ATCC 22284	CM
Thraustotheca	*T. clavata* ATCC 14555	YM
Pythium	*Pythium* sp. ATCC 11270	CM
Pythium	*P. debaryanum* ATCC 9998	YM
Pythium	*P. irregulare* ATCC 10951	YM
Pythium	*P. ultimum* ATCC 11123	YM
Subdivision: *Zygomycotina*		
Conidiobolus	*C. coronatus* NRRL[b] 1912	PO
Conidiobolus	*C. coronatus* ATCC 24160	CM
Conidiobolus	*C. obscurus* ARS[c] 133	SA
Conidiobolus	*C. thromboides* ARS 115	SA
Zoophthora	*Z. radicans* ARS 141	SA
Subdivision: *Ascomycotina*		
Lipomyces	*L. lipofer* NRRL 1351	YM
Lipomyces	*L. lipofer* NRRL 6333	YM
Subdivision: *Deuteromycotina*		
Geotrichum	*G. candidum* ATCC 4798	YM
Geotrichum	*G. candidum* NRRL 552	YM

[a] ATCC, American Type Culture Collection, Rockville, MD.
[b] NRRL, Northern Regional Research Center, Peoria, IL.
[c] ARS, Plant Protection Research Laboratory, USDA/ARS/NAA, Cornell University, Ithaca, NY.

on Corn Meal Agar (Difco Laboratories, Detroit, MI). Organisms of the genera *Lipomyces* and *Geotrichum* were maintained on Yeast Maintenance Agar (Difco). *C. coronatus* NRRL 1912 was maintained on Potato Dextrose Agar (Difco). *C. obscurus*, *C. thromboides* and *Z. radicans* were maintained on media containing 3.0 g/l KH_2PO_4 (J.T. Baker and Co., Phillipsburg, NJ), 3.0 g/l yeast extract (Difco), 10.0 g/l neopeptone (Difco), 10.0 g/l D-glucose (Sigma Chemical Co., St. Louis, MO) and 20.0 g/l agar (Difco).

Growth conditions. Experiments were conducted using 100 ml of media in 250 ml shake flasks (Erlenmeyer) which were agitated at 135 rpm. *C. obscurus*, *C. thromboides* and *Z. radicans* were grown at 20 °C; all other organisms were grown at 24 °C. Inocula were grown in media specified in Table 1 plus 10.0 g/l D-glucose; media ingredients are listed in Table 2. Inoculum growth was initiated by transferring culture from a maintenance slant. After an inoculum became visibly dense, it was macerated for 30 s at low speed using a 300 ml macerator (Eberbach Corp., Ann Arbor, MI) mounted on a commercial blender (Waring Products, Hartford, CT). Two ml of inoculum was transferred to shake flasks used for experimental studies.

Dry weight assay. Dry weight concentrations for first, second and fourth level experiments were determined by filtering 10 ml of macerated fermentation broth through an 0.45 μm pre-dried and tared Super 450 filter disc (Gelman Sciences, Ann Arbor, MI). The filter mat was washed with distilled water and dried at 100 °C for 24 h. Dry weight concentrations were measured after the filter discs were allowed to cool in a desiccator. For co-chromatography and GC/MS samples of the third level experiments, approximately 1.0 l of culture was filtered through a coarse sintered-glass filter funnel and washed. The filter

TABLE 2

Media ingredients

CM medium: Corn meal infusion broth 1.0 l

Corn meal infusion broth recipe

1. Soak 50 g corn meal (Quaker yellow corn meal) in 800 ml distilled water for 5 h in 4 °C refrigerator
2. Heat for 1 h at 60 °C
3. Place in refrigerator overnight
4. Vacuum filter through cheesecloth while cold
5. Vacuum filter through Shark-Skin filter paper
6. Gravity filter through Reeve Angele no. 802 filter paper
7. Vacuum filter through no. 50 Whatman filter paper
8. Bring volume up to 1.0 l with distilled water

PO medium: Patato infusion broth 1.0 l

Patato infusion broth recipe

1. Cook 300 g finely diced potatoes (skinned) in 500 ml distilled water for approximately 40 min (until soft)
2. Gravity filter through cheesecloth
3. Refrigerate overnight
4. Centrifuge at 10000 × *g* for 5 min
5. Vacuum filter through no. 50 Whatman filter paper
6. Bring volume up to 1.0 l with distilled water

SA medium:	Yeast extract	3.0 g
	neopeptone	10.0 g
	KH_2PO_4	3.0 g
	distilled water	1.0 l
YM medium:	yeast extract	3.0 g
	malt extract (Difco)	3.0 g
	peptone (Difco)	5.0 g
	distilled water	1.0 l

mat was lyophilized for 72 h and weighed to determine the dry weight concentration.

Lactose, glucose and galactose assays. Samples were filtered through 25 mm nylon 66 syringe filters having an 0.45 μm nominal pore diameter (Rainin Instrument Co., Inc., Woburn, MA). Filtrates were diluted with the mobile phase when concentrations exceeded 1.0 g/l and concentrated under a stream of nitrogen when concentrations were less than 0.1 g/l.

The HPLC unit consisted of Spectra Physics HPLC modules (San Jose, CA). The specific modules were an autosampler SP8780 XR with a 50 μl sample loop, mobile phase pump and pump control unit SP8700, differential refractometer SP6040, and strip-chart printer/integrator SP4270. The column was an Aminex ion exclusion column, HPX-87H, 300 × 7.8 mm (Bio-Rad Laboratories, Richmond, CA), fitted with a Cation-H guard column, 40 × 4.6 mm. The mobile phase was helium-sparged 0.3 M HNO_3 with a flow rate of 0.3 ml/min. Lactose, glucose and galactose concentrations were measured by peak area mode. Standard mixtures, used for identification purposes, were made from triple deionized water, and anhydrous α-lactose, D-glucose and D-galactose (Sigma).

EPA assay. Macerated culture samples from the first, second and fourth level experiments and lyophilized culture samples from the third level experiments were extracted with $CHCl_3 : CH_3OH$ (2 : 1 v/v) via the Folch Method [5]. The lipid phase of the Folch extract was evaporated to dryness under a stream of nitrogen and weighed. This crude lipid was dissolved in $CHCl_3$ (approximate concentration of 20 mg/ml). A 1.0 ml crude lipid sample was hydrolyzed and methylated as described by Slover and Lanza [9]. Heptadecanoic acid was used as the internal standard for GC quantification of EPA.

GC was performed using a Hewlett Packard HP 5890 GC (Avondale, PA) equipped with a flame ionization detector (FID), a split/splitless injection system and a Supelcowax 10 capillary column, 30 m length × 0.32 mm i.d., having a 0.25 μm film thickness (Supelco Chromatography Products, Belefonte, PA). The column oven was programmed for 180 °C isothermal operation for 16 min followed by a 3 °C/min temperature program to 240 °C final temperature. The detector and injector temperatures were set at 220 °C and the carrier gas (helium) flow rate was approximately 0.92 ml/min. Operating in the split injection mode, the solvent purge rate was 1.0 ml/min and the split vent purge rate was approximately 136 ml/min. Chromatograms were recorded on an HP 3393 A integrator. Standard mixtures used for identification purposes were PUFAs obtained from Supelco Chromatography Products, and fatty acid and FAME standards obtained from Sigma Chemical Co.

EPA identification was based on GC retention times (R_t). This required further analysis before an organism could be verified as an EPA producer. The verification process included co-chromatography of culture samples and culture samples spiked with standard methyl EPA, and GC/MS. EPA was identified if the standard methyl EPA co-eluted with the material expected to be methyl EPA, and the calculated amount of total methyl EPA in the spiked sample equaled the measured amount; the calculated amount was based on the amount measured in the sample not spiked and the amount added to the spiked sample. EPA production was verified with GC/MS by measuring the molecular weight of the compound identified as EPA. GC/MS was performed using an HP 5992 GC/MS instrument operated without FID. Column and oven conditions were the same as for GC. The transfer line temperature to the mass spectrometer was 240 °C. The mass analyzer and ion source temperature was 200 °C. The injection port temperature was 250 °C.

First level experiments. Each organism was grown in three types of media. The first medium is specified for a particular organism in Table 1; media ingredients are listed in Table 2. The second medium was the first medium plus 9.5 g/l α-lactose (Sigma). The third medium was the first medium plus 10.0 g/l D-glucose. These glucose and lactose concentrations are equivalent on the basis of carbon concentration. When one of the three cultures had a rising or high pH, all three cultures were analyzed for dry weight yield. Media and media plus lactose were used to test for lactose utilization. An organism utilizing lactose should have a higher final dry weight when grown for several days on the medium with lactose than on the medium alone, the negative control. Organisms were grown on media with glucose as a positive control; this was to confirm that lactose, not the media, was growth limiting, if the final dry weight for growth on media with lactose was the same as for growth on media alone.

Second level experiments. Frozen samples were analyzed for lactose and glucose utilization with HPLC and EPA production with GC if dry weight results indicated lactose utilization.

Third level experiments. These experiments were conducted on organisms which had lactose utilization confirmed by HPLC and EPA production indicated by GC. A SWP medium was first developed for each; these media are listed in Table 3. Organisms were then grown in SWP media where dry weight measurements were used to identify SWP utilizers. Verification of EPA production was tested by co-chromatography and GC/MS.

Fourth level experiments. Organisms which used SWP and had EPA production confirmed were examined in growth studies. The submerged cultures grown here were

TABLE 3

SWP media

Medium Name		Medium
SY	SWP	10.0 g
	yeast extract	3.0 g
	KH_2PO_4	3.0 g
	distilled water	1.0 l
(adjust pH to 6.0 with 2 M KOH before sterilizing)		
SYMP	SWP	10.0 g
	yeast extract	3.0 g
	malt extract	3.0 g
	peptone	5.0 g
	KH_2PO_4	3.0 g
	distilled water	1.0 l
(adjust pH to 6.0 with 2 M KOH before sterilizing)		
SYN	SWP	10.0 g
	yeast extract	3.0 g
	neopeptone	10.0 g
	KH_2PO_4	3.0 g
	distilled water	1.0 l
(adjusted pH to 6.0 with 2 M KOH before sterilizing)		

not homogeneous. Lactose, glucose, galactose, dry weight, pH and EPA profiles were obtained by inoculating 8 to 10 shake flasks containing a SWP medium. Flasks were removed sequentially over a period of several days for analysis.

TABLE 4

Dry weight concentration results for first level experiments where CM medium, CM medium with lactose and CM medium with glucose were used

Organism	Dry weight concentration (g/l)		
	O[a]	L[b]	G[c]
Subdivision: *Mastigomycotina*			
A. bonariensis ATCC 22407	0.4	0.5	1.9
S. parasitica ATCC 22284	0.4	0.5	1.5
Subdivision: *Zygomycotina*			
C. coronatus ATCC 24160	0.3	0.3	1.1
Pythium sp. ATCC 11270	0.4	0.9	1.4
Control[d]	0.0	0.0	0.0

[a] O, CM medium was used.
[b] L, CM medium with lactose was used.
[c] G, CM medium with glucose was used.
[d] Uninoculated medium.

TABLE 5

Dry weight concentration results for first level experiments where SA medium, SA medium with lactose and SA medium with glucose were used

Organism	Dry weight concentration (g/l)		
	O[a]	L[b]	G[c]
Subdivision: *Zygomycotina*			
C. obscurus ARS 133	1.6	3.0	5.1
C. thromboides ARS 115	5.2	5.1	9.5
Z. radicans ARS 141	1.6	1.5	7.2
Control[d]	0.0	0.1	0.1

[a] O, SA medium was used.
[b] L, SA medium with lactose was used.
[c] G, SA medium with glucose was used.
[d] Uninoculated medium.

RESULTS

Tables 4 through 7 contain the dry weight results of the first level experiments. An organism having a dry weight yield on medium plus lactose that was at least 0.2 g/l greater than on medium alone, was tentatively classified as a lactose utilizer; 9 organisms were identified. Examination of controls shows that the media contribute no more than 0.1 g/l dry weight. The dry weight yield on media plus glucose was greater than on media plus lactose; this verified that lactose, and not the media, was growth limiting.

Table 8 shows that *L. lipofer* NRRL 1351 and *L. lipofer* NRRL 6333 did not produce EPA. The remaining seven organisms produced EPA, utilized 1.3 to 9.6 g/l lactose and 5.5 to 11.6 g/l glucose. This, in conjunction with the

TABLE 6

Dry weight concentration results for first level experiments where PO medium, PO medium with lactose and PO medium with glucose were used

Organism	Dry weight concentration (g/l)		
	O[a]	L[b]	G[c]
Subdivision: *Zygomycotina*			
C. coronatus NRRL 1912	1.0	1.2	4.3
Control[d]	0.0	0.0	0.0

[a] O, PO medium was used.
[b] L, PO medium with lactose was used.
[c] G, PO medium with glucose was used.
[d] Uninoculated medium.

TABLE 7

Dry weight concentration results for first level experiments where YM medium, YM medium with lactose and YM medium with glucose were used

Organism	Dry weight concentration (g/l)		
	O[a]	L[b]	G[c]
Subdivision: *Mastigomycotina*			
A. echinulata ATCC 22408	1.9	2.0	6.6
D. monosporus ATCC 34931	1.2	1.0	6.6
T. clavata ATCC 14555	2.2	2.6	6.1
P. debaryanum ATCC 9998	2.0	2.8	5.5
P. irregulare ATCC 10951	1.4	2.0	3.3
P. ultimum ATCC 11123	1.6	1.9	4.7
Subdivision: *Ascomycotina*			
L. lipofer NRRL 1351	1.4	4.6	5.0
L. lipofer NRRL 6333	1.4	2.0	4.0
Subdivision: *Deuteromycotina*			
G. candidum ATCC 4798	1.2	1.2	5.8
G. candidum NRRL 552	1.3	1.3	4.0
Control[d]	0.1	0.1	0.0

[a] O, YM medium was used.
[b] L, YM medium with lactose was used.
[c] G, YM medium with glucose was used.
[d] Uninoculated medium.

dry weight results, shows that glucose was used more efficiently than lactose, but lactose was used.

Table 9 specifies the SWP medium used for each organism. Table 9 shows that the final dry weight of the *C. obscurus* culture grown in control medium is greater than the final dry weight for the *C. obscurus* culture grown in SWP medium; hence it does not utilize SWP. The difference in dry weight yields for *T. clavata* and *C. coronatus* grown on SWP media vs. control media was small (≤ 0.4 g/l). The difference in dry weight yields of the remaining four organisms grown on SWP media vs. control media was larger (≥ 0.9 g/l). These results suggest that *Pythium* sp. ATCC 11270, *P. debaryanum*, *P. irregulare* and *P. ultimum* are the most efficient SWP users.

TABLE 9

Dry weight concentration results for the third level experiments

Organism	Dry weight concentration (g/l)		SWP medium
	Control[a]	SWP[b]	
Pythium sp. ATCC 11270	i[c]	0.9	SY
P. debaryanum ATCC 9998	i	1.7	SY
C. coronatus NRRL 1912	3.9	4.3	SYN
P. irregulare ATCC 10951	i	3.3	SYMP
P. ultimum ATCC 11123	i	4.0	SYMP
T. clavata ATCC 14555	3.7	4.0	SYMP
C. obscurus ARS 133	3.8	2.9	SYN

[a] Control designates that the medium used was the same as the SWP media but without the SWP.
[b] SWP, the medium used was SWP medium.
[c] i, growth was insufficient to determine dry weight concentrations.

TABLE 8

Results for second level experiments showing the amount of EPA produced, amount of lactose utilized for growth on media with lactose, and glucose utilized for growth on media with glucose

Organism	EPA[a] (mg/l)		Lactose[a] (g/l)	Glucose[a] (g/l)
	L[b]	G[b]	L	G
Pythium sp. ATCC 11270	12	44	4.6	8.7
T. clavata ATCC 14555	3	4	6.0	8.9
P. debaryanum ATCC 9998	13	31	9.4	8.9
P. irregulare ATCC 10951	10	14	9.3	8.8
P. ultimum ATCC 11123	11	30	5.1	8.6
L. lipofer NRRL 1351	0	0	9.6	8.9
L. lipofer NRRL 6333	0	0	3.2	8.9
C. coronatus NRRL 1912	0	1	1.3	11.6
C. obscurus ARS 133	23	4	5.9	8.6

[a] The amount of lactose and glucose utilized was calculated by substracting the amount in growth flasks from the amount in uninoculated flasks of the same medium; EPA was never detected in uninoculated media.
[b] L, media with lactose was used.
[c] G, media with glucose was used.

All uninoculated SWP media tested negative for EPA, and in all SWP-media cultures the product was identified as EPA using co-chromatography and verified as EPA with GC/MS by giving an apparent molecular weight of 316 for methyl EPA.

Fig. 1 shows the lactose, glucose and galactose curves for *Pythium* sp. ATCC 11270, *P. debaryanum*, *P. irregulare* and *P. ultimum*. The rate of lactose disappearance, based on the initial and final lactose values, was low for *Pythium* sp. ATCC 11270 (0.025 g/l/h) when compared to the other three organisms. Over this time period, 1.1 g/l glucose and 1.0 g/l galactose were produced and 2.5 g/l lactose disappeared. Because hydrolysis of 2.5 g/l lactose yields ca. 1.3 g/l galactose and ca. 1.3 g/l glucose, these results suggest that *Pythium* sp. ATCC 11270 used little, if any, glucose or galactose. The rates of lactose disappearance for *P. debaryanum*, *P. irregulare* and *P. ultimum*, based on initial and final lactose values, were 0.069, 0.063 and 0.050 g/l/h, respectively.

Fig. 1 shows that for all except *Pythium* sp. ATCC 11270, galactose and glucose are produced as lactose disappears, and the glucose produced is used. Based on lactose disappearance, the theoretical amount of galactose formed compared to measured values was 3.7 : 3.3 g/l for *P. debaryanum*, 3.0 : 2.3 g/l for *P. irregulare* and 2.6 : 1.9 g/l for *P. ultimum*. These results suggest that these organisms also used galactose.

Fig. 2 shows that all four organisms had an increasing pH throughout the total time period; this suggests that the protein in SWP was also being used. *Pythium* sp. ATCC 11270 is shown in Fig. 2 to have the lowest maximum dry weight concentration of 0.8 g/l and lowest maximum EPA concentration of 5.0 mg/l. *P. debaryanum* had the highest maximum dry weight concentration of 4.2 g/l and a maximum EPA concentration of 15.3 mg/l. *P. irregulare* gave a maximum dry weight concentration of 2.1 g/l and an EPA concentration of 14.5 mg/l. *P. ultimum* had a maximum

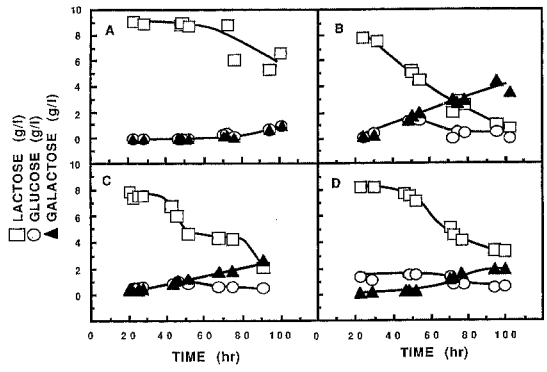

Fig. 1. Lactose, glucose and galactose curves for fourth level experiments. (A) *Pythium* sp. ATCC 11270; (B) *P. debaryanum*; (C) *P. irregulare*; (D) *P. ultimum*.

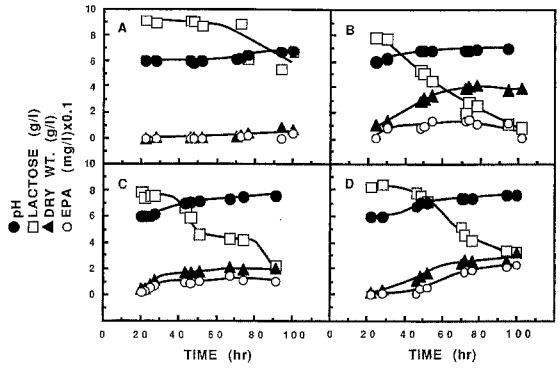

Fig. 2. Fourth level experimental results showing pH, lactose, dry weight and EPA curves. (A) *Pythium* sp. ATCC 11270; (B) *P. debaryanum*; (C) *P. irregulare*; (D) *P. ultimum*.

dry weight concentration of 3.2 g/l and the largest maximum EPA concentration of 22.8 mg/l.

P. debaryanum curves do not show an EPA concentration increasing with dry weight concentration as the other three organisms do. This indicates that EPA production is not growth associated for *P. debarynum*. *Pythium* sp. ATCC 11270 and *P. ultimum* show lactose utilization rates that are approximately inversely proportional to dry weight and EPA production rates. *P. irregulare* does not demonstrate either of these characteristics. *P. debaryanum* demonstrates a lactose utilization rate that is only approximately inversely proportional to dry weight.

DISCUSSION

The purpose of this work was to identify organisms which use SWP and produce EPA. In the U.S. and other dairy producing countries a large volume of sweet whey is currently not utilized. EPA is of interest because of its potential pharmaceutical value.

The four-level protocol provided a means of eliminating organisms in a sequential process. The first level identified 9 of the 18 original organisms as possible lactose users. The second level demonstrated that 7 of these had both lactose utilization confirmed by HPLC and EPA production indicated by GC. In the third level it was shown that only 4 (*Pythium* sp. ATCC 11270, *P. debaryanum*, *P. irregulare* and *P. ultimum*) best used SWP and had EPA production verified by co-chromatography and GC/MS. These organisms are from only one of the four subdivisions sampled, *Mastigomycotina*.

Growth studies conducted in the fourth level experiments on *Pythium* sp. ATCC 11270, *P. debaryanum*, *P. irregulare* and *P. ultimum* were directed at determining which had the best dry weight and EPA yield, and best utilized the lactose in SWP for dry weight and EPA production. *P. ultimum* is the best overall candidate for future

study because it produced the highest concentration of EPA, a good dry weight concentration, had growth associated EPA production, and lactose appeared to be used for dry weight and EPA production.

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