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(54) Title: METHOD TO PRODUCE SINGLE CELL OIL CONTAINING GAMMA-LINOLENIC ACID

(57) Abstract

The invention discloses a method for producing a single cell oil containing gamma-linolenic acid characterised in that at least one microorganism of the order *Mucorales* preferably of the genus *Mortierella*, *Actinomucor*, *Mucor*, *Rhizomucor* or *Rhizopus* is cultured in a growth medium which is substantially starch and sugar free and which contains, as a carbon source material, at least one monocarboxylic acid of 2 to 5 carbon atoms, preferably acetic acid and recovering the oil from the resultant cultured microorganism biomass. The invention also relates to a method of creating the organic acid stream of the Fischer-Tropsch synthesis process to remove organic material therefrom.

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METHOD TO PRODUCE SINGLE CELL OIL CONTAINING GAMMA-LINOLENIC ACID

THIS invention relates to a biological method of treating industrial effluent carrying carbon containing chemicals but which is substantially devoid of starches and sugars. It also relates to a method of cultivating microorganisms and to the recovery of valuable metabolites therefrom.

BACKGROUND OF THE INVENTION

Carbon based industrial chemical processes often give rise to aqueous effluent streams carrying a variety of carbon containing compounds. Thus, for example, it is known that the Synthol or Fischer-Tropsch Synthesis in which hydrocarbons, aliphatic alcohols, aldehydes and ketones are produced by the catalytic hydrogenations of carbon monoxide gives rise to an aqueous by-product or effluent stream, known as the "Fischer Tropsch organic acid stream". This stream typically contains between 1% and 3% of C_2 to C_5 monocarboxylic acids along with non-acidic chemicals such as ketones and aldehydes. This stream is conventionally treated by an activated sludge to strip the stream of its carbon content before recycling the purified water into the cooling circuit of the plant or running it into a river or the sea. The resultant biomass is either incinerated or may be disposed of as fertilizer.

In an unrelated field of technology it is known that certain microorganisms, known as oleaginous organisms, are capable of producing edible oils which oils have become known as Single Cell Oils or SCO's. [See for example the overview SINGLE CELL OIL edited by R.S. Moreton, Longman Scientific & Technical, 1988]

According to the work of Moreton referred to above, the first truly commercial SCO process is the one being carried out at the plant of Sturge Biochemicals in the United Kingdom at which a lipid rich in gamma linolenic acid [GLA; 6,9,12-octadecatrienoic acid] is produced. According to a report by K.W. Sinden of John and E. Sturge Limited [*Enzyme Microb. Technol.*, 1987 Vol. 9 p. 124-125] the microbial oil rich in GLA is produced by a *Mucor sp.* cultivated on a pure defined substrate based on glucose.

Gamma Linolenic Acid is a high value product which occurs in several natural products including breast milk, evening primrose oil, oats and other products. In the human and animal body it is converted to prostaglandin E_1, one of the important localised hormone type products regulating the body functions of the kidneys, liver, lungs, brain, nerve system and immune system. Products containing GLA are presently widely used in many parts of the world as a component of health food programs.

A large number of oleaginous organisms has been reported in the literature. The feedstock range on which such oleaginous organisms may be cultivated to produce lipids is quite diverse. According to literature reports such feedstock ranges from molasses, bananas, whey and potato starch to exotic carbon sources such as pentose, hexose sugars, disaccharides, glycerol, amino acids and ethanol. In general all such organisms are capable of assimilating glucose and other sugars while some are capable of assimilating starches. Reports on microorganisms capable of being cultivated on simpler forms of carbon-containing chemicals such as ethanol and glycerol do exist but relate to only a small number of specific SCO producing organisms.

It has been reported in Japanese patent application 81012479 that the microorganisms *Mucor javanicus*, *Rhizopus delemar* and *Aspergillus asumi* mut. *shiro-usamii* may be cultivated on waste water discharged from brewing, starch, rice, confectionery and cake producing or food processing factories. It will be readily appreciated that such waste water contains starch and/or sugars which are known nutrients for sustaining the growth of those microorganisms.

It has further been reported in European Patent Application 0269351 in the name of Lion Corporation that certain gamma-linolenic acid producing micro-organisms may be cultured with fatty acids or fatty acid esters, more particularly fatty acids of between 8 and 22 carbon atoms as carbon source for the production of gamma-linolenic acid.

In European Patent Application 0155420 it is reported that microorganisms of the Mortierella genus may be cultured on glucose to produce a lipid rich in gamma-linolenic acid and that the efficiency of the culture is enhanced by the addition of acetic acid or an alkali metal acetate.

From the aforegoing it will be seen that the search for alternative producers of lipids rich in gamma-linolenic acid and for alternative feedstocks for use as a carbon source for such organisms is ongoing as it would clearly be advantageous to provide a process by which lipids rich in gamma-linolenic acid may be produced from simpler forms of carbon source materials.

It has now been found that certain non-starch and non-sugar carbon containing compositions based on mono carboxylic acids having between 2 and 5 carbon atoms, and in particular acetic acid, are capable of being used as a feedstock for the cultivation of certain oleaginous microorganisms capable of producing valuable Single Cell Oils. This unpredictable finding is made even more surprising by the fact that the feedstock in question does not sustain the growth of all oleaginous microorganisms. Furthermore, such feedstock, according to presently available results, does not appear to sustain growth of the organisms in question throughout their entire life cycle. Although the organisms germinate from spores introduced into the feedstock and develop to the hyphal stage, the organisms do not generally proceed to the stage of sporulation with the result that the feedstock in question would not appear to be capable of constituting a natural ongoing habitat for the organisms capable of sustaining successive life cycles until the carbon source is depleted. Despite the incomplete life cycle we have found that the organisms in question are capable of converting the simple chemical compounds present in the feedstock in question into high value oils and other chemical products.

It has further been found that the Fischer-Tropsch organic acid stream, which contains C_2 to C_5 mono-carboxylic acids can be used as a feedstock carbon source for the cultivation of certain oleaginous microorganisms.

OBJECT OF THE INVENTION

It is accordingly an object of the present invention to provide a method of cultivating certain SCO producing microorganisms in a new feedstock composition and to recover valuable lipids from the cultured biomass. It is a further object of the invention to provide a method of treating an effluent stream to remove certain carbon-based chemicals therefrom.

GENERAL DESCRIPTION OF THE INVENTION

According to the invention there is provided a method for producing a single cell oil containing gamma-linolenic acid characterised in that at least one microorganism of the order Mucorales is cultured in a growth medium which contains, as a carbon source material, at least one monocarboxylic acid of between 2 and 5 carbon atoms and which is substantially starch and sugar free, and recovering the oil from the resultant cultured microorganism biomass.

In one form of the invention the microorganism may be of the family Mortierellaceae, preferably of the genus Mortierella and most preferably selected from the species consisting of Mo. isabellina, Mo. longicollis and Mo. ramanniana var. ramanniana.

In an alternative form of the invention the microorganism is preferably of the family Mucoraceae.

Thus the microorganism is preferably selected from the group consisting of the genera Actinomucor, Mucor, Rhizomucor and Rhizopus.

In this form of the invention the microorganism is most preferably selected from the group consisting of the following species of the genus Mucor, namely:

Mu. amphibiorum
Mu. ardhlaengiktus
Mu. azygosporus
Mu. bainieri
Mu. circinelloides f. griseocyanus
Mu. circinelloides f. circinelloides
Mu. circinelloides f. janssenii
Mu. circinelloides f. lusitanicus
Mu. fragilis
Mu. fuscus
Mu. hiemalis f. hiemalis
Mu. minutus
Mu. mousanensis
Mu. oblongisporus
Mu. plumbeus
Mu. prayagensis
Mu. recurvus var. indicus
Mu. recurvus var. recurvus
Mu. rouxii
Mu. sinensis
Mu. subtilissimus
Mu. tuberculosporus
Mu. variabilis
Mu. variosporus
Mu. zychae var. zychae

In the most preferred form of the invention the microorganisms may comprise Mucor javanicus whemer [described in Zentbl. Bakt. Parasit Kde. Abt., 2, 6:619, 1900] which was originally isolated from "Chinese yeast" [Ragi] at Java, Indonesia. The type culture of this organism is available as Mucor *circinelloides* f. *circinelloides*, CBS 203.28 from the open collection of Centraalbureau voor Schimmelcultures in Baarn, Netherlands. Another most preferred organism is *Mucor rouxii* which is similarly available from CBS and which is deposited in the open collection of that culture collection under number CBS 416.77.

The organic material in the growth medium may constitute between 0,5% and 10%, but preferably between 1% and 3% of the medium.

In a particular form of the invention the growth medium may comprise the organic acid stream derived from a Fischer-Tropsch Synthesis process and may typically have the following composition on a mass/mass basis:

CH_3COOH - between 0,6% and 1,15%
C_2H_5COOH - between 0,2% and 0,4%
i-C_3H_7COOH - between 0,06% and 0,19%
n-C_3H_7COOH - between 0,09% and 0,40%
i-C_4H_9COOH - between 0,02% and 0,10%
n-C_4H_9COOH - between 0,03% and 0,05%
balance of organic compounds - between 0,02% to 0,04%
inorganic materials - less than 0,006%
water - balance to 100%

The total acid content of the medium preferably does not exceed 10 g/l.

Preferably the organic acid medium is modified to contain assimilable nutrient sources approximately in the ratio

C : N : P of 100 : 14 : 4.

The nutrient mixture preferably further contains assimilable sources of potassium and sulphur, preferably in the form of sulphates, which are present approximately in the ratio

C : N : P : K : S of 100 : 14 : 4 : 3 : 1

The nitrogen may be reduced to a ratio C : N of 100 : 2 once the organism has reached the early to middle exponential growth phase.

The assimilable nitrogen and phosphate nutrients added to the effluent are of course only necessary insofar as the medium itself if derived from an industrial process may lack such nutrients. Where required to be added the primary nutrients may generally be added in the form of ammonium hydroxide and phosphoric acid. Phosphate salts may also be added to the growth medium.

Should the medium not contain quantities of trace elements necessary to sustain growth, a suitable cocktail of such trace elements may be added to the growth medium. In this regard it has been found that a growth medium as described above and which in addition contains the elements As, Hg, Li, Mn, Cr, Cu, in quantities of less than 0,1 mg/l;

the elements Cd, Co, Zn and Fe in quantities of between 0,1 and 1 mg/l;
and the elements Na, Ca, Si, Cl, Pb, Ni, F in quantities of between 1 and 10 mg/l, is suitable for sustaining growth of the microorganisms in question.

In culturing the organisms the growth medium is preferably incolulated by suspending a quantity in excess of 10^4 spores per litre of medium onto the medium. Most preferably the quantity is not in excess of 10^6 spores per litre. The spores are preferably applied to the effluent in the form of a powdered mixture of a sugar and/or starch-based source material on which the inoculant had been cultured to the sporulation stage.

The incubation of the inoculated growth medium is preferably carried out for a period of between 40 and 120 hours at a temperature of between 25° and 32°C, most preferably at 30°C with aeration to maintain a dissolved oxygen level of 0,5 to 3,5 and preferably about 2,0 to 2,5 mg/litre in the growth medium, until the spores had developed to a biomass of the hyphal stage in the early to middle exponential growth phase of the organism. Nitrogen supply is then reduced. The biomass is thereafter separated from the medium when the organisms have reached the stationary growth phase.

The separation of the biomass and the water after incubation may be carried out in any convenient manner, e.g. by filtration.

The separated biomass may thereafter be extracted to recover the SCO's therefrom. The extraction is preferably carried out with hexane or chloroform and the solvent fraction may thereafter be separated into its component parts by fractional distillation.

The extracted biomass residue, which is rich in protein may be used as monogastric animal fodder, e.g. chicken feed.

Where the process of the present invention is carried out for the dual purpose of producing SCO's and for removing of organic material from the Fischer-Tropsch organic acid stream, the water fraction, after removal of the biomass and hence substantially depleted of carbon-containing material, may, if necessary, be polished to remove excess phosphates and/or ammonia if the stream is to go to waste. The stream is however preferably re-circulated as diluent water.

The harvested biomass may be treated after harvesting to recover SCO's, squalene and/or chitosan therefrom.

Examples of the invention will now be described without thereby limiting the scope of the invention to the described embodiments.

E X A M P L E 1

Culturing of **Mucor javanicus** with Fischer-Tropsch organic acid water

A sample of the organic acid stream of a Fischer-Tropsch synthetiser was analysed and found to contain approximately 1,4% [m/m] mono-carboxylic acids as major ingredients in water. The acids were present in approximately the following ratio:

CH_3COOH	-	30 parts
C_2H_5COOH	-	10 parts
$i\text{-}C_3H_7COOH$	-	2,00 parts
$n\text{-}C_3H_7COOH$	-	4,00 parts
$i\text{-}C_4H_9COOH$	-	1,00 part
$n\text{-}C_4H_9COOH$	-	1,00 part.

The acid effluent also contained other so-called non-acid chemicals which were present in much smaller quantities and which collectively constituted only about 0,04% of the effluent. The acid effluent also contained about 0,006% of inorganic matter.

The sample was diluted to an acid concentration of 4000 mg/litre by the addition of water and placed in a 17 litre fermenter. The fermenter was maintained at 30°C and inoculated with 17g of <u>Mucor javanicus</u> spores prepared as described in Example 2 below, each gram of the inoculant powder containing about 10^9 spores. The following nutrients were also added to the mixture:

$MgSO_4.7H_2O$ - 0,765 grams
K_2HPO_4 - 1,785 grams
NH_3(25%) - 20 ml
H_3PO_4(85%) - 1,7 ml

The mixture was mixed and incubated with aeration to maintain a dissolved oxygen level of 2,0 mg/litre at the above temperature for 24 hours. The aeration is preferably carried out so as to establish airlift and so that the introduced air also serves to mix the culture medium. The pH of the mixture was maintained at pH 5.8 by the addition on demand of fresh feedstock or, if necessary 5 Molar NaOH. It has been found advantageous to conduct the fermentation on this combination of a fed batch, pH controlled, airlift fermentation basis. Part of the resulting biomass was separated from the fermentation broth with the aid of sieves, dried and weighed.

The dry biomass was extracted with hexane, and the hexane was removed to obtain 5,5 grams of a crude oil fraction per 100g of dry biomass.

The crude oil fraction was analysed and found to contain *inter alia* C_{16} and C_{18} fatty acids.

The fatty acid composition of the crude oil fraction was shown by GC analysis to comprise:

25%	$C_{16:0}$	[Palmitic acid]
1,7%	$C_{16:1}$	[Palmitoleic acid]
2,1%	$C_{18:0}$	[Stearic acid]
24,6%	$C_{18:1}$	[Oleic acid]
23,6%	$C_{18:2}$	[Linoleic acid]
22,9%	$C_{18:3}$	[Gamma-Linolenic acid]

The oil fraction may of course be distilled to obtain the constituents separate from one another. Squalene and chitosan may also be recovered from the biomass.

EXAMPLE 2

The spores for use in inoculating the growth medium as described in Example 1 above was prepared by inoculating a starch matrix made from wheat flour with spores of *Mucor javanicus*. The inoculated material was left in a damp atmosphere for 3 days at 30°C during which period the microorganism grew vigorously, to the substantial exclusion of other organisms, and to the stage of sporulation.

The matrix was dried and milled to a fine powder. A spore count was performed and it was found that the powder contained about 10^9 spores per gram.

EXAMPLE 3

In order to demonstrate that the ability to convert the simple monocarboxylic acids present in the Fischer-Tropsch organic acid water [FT water] to lipids containing gamma-linolenic acid [GLA] is shared by a large number of species in the order Mucorales, a number of strains belonging to the genera Actinomucor, Mortierella, Mucor, Rhizomucor and Rhizopus were obtained from the Centraalbureau voor Schimmelcultures, Delft [The Netherlands] and maintained on YM agar slants [Wickerham, 1951] at 21°C.

A loopful of spores were used to inoculate 200 ml of a culture medium in 500 ml conical flasks, after which it was incubated on a shaker [150 rpm] at 30°C. The culture medium contained 0,10 g/l Yeast extract, 0,25 g/l $MgSO_4.7H_2O$, 10,00 g/l K_2HPO_4, 0,62 g/l NH_3, 0,05 g/l $CaCl_2.2H_2O$ and 333 ml/l FT water, giving 4 g/l total acids. The pH was set at 5.8.

It was observed that some of the strains tested for growth did not grow in the medium. Of the strains investigated the 40 best performers in terms of growth were re-tested by using 100 ml culture medium in

1000 ml conical flasks. Three repetitions of each culture were performed and the cultures were harvested at three different times. It was observed that GLA production was dependent on the duration of cultivation, the concentration increasing with time to a maximum and then decreasing thereafter.

Harvesting: The cultures were harvested by filtration through Whatman No. 1 filter paper, after which the cellular material was freeze dried.

Fatty acid analyses using gas chromatography [Megabore column] [Kock et al, 1985]. To 0,12 g lyophilised fungal material in a screw-capped [teflon-lined] glass tube was added 5,0 ml of 15% KOH in 50% CH_3OH/H_2O. Each tube also received 30 microlitre 6% lauric acid [12:0] in methanol as internal standard. The tubes were sealed and heated in a boiling waterbath for 1 hour with continuous shaking. After cooling to room temperature the contents were adjusted to pH 2 by addition of 1,5 ml 32% HCl. After addition of 3,0 ml 20% BF_3/CH_3OH complex [Merck, Darmstadt], and flushing with N_2, each tube was re-sealed and heated in a boiling waterbath for 15 minutes with continuous shaking. Each reaction mixture was again cooled to room temperature, 0,25 ml saturated NaCl solution was added and the methylated fatty acids were extracted with three successive 6,0 ml aliquots of 1:4 $CHCl_3/C_6H_{14}$ [hexane]. The combined extracts were concentrated under a slow stream of N_2, the methylated fatty acids were re-dissolved in 1,8 ml C_6H_{14} and transferred to a 2 ml glass vial equipped with a teflon-lined screw-cap.

Gas chromatography was carried out with a Varian 3300 gas chromatograph equipped with a FID detector. A polar Supelcowax 10 column [30 m x 0,75 mm, inside diameter] and N_2 as carried gas [flowrate, 5 ml/min] were used for separation.

The GLA content [mg/g lyophilised fungal material] was calculated by using the surface area of the peak from the internal standard [12:0] on the gas chromatogram as response reference.

The results of these determinations are set out in Table 1 below in which the highest observed GLA production is recorded against each of the best performing strains as well as the time at which it was harvested. Different yields were obtained at different harvesting times. The CBS number under which the various strains are available from the open collection of CBS in Baarn, Netherlands are also given in Table 1.

TABLE 1

Species	CBS	mgGLA/ g	Harvest time hours
Genus : Actinomucor			
Actinomucor elegans	100.09	16.27	44
Genus: Mortierella			
Mo. isabellina	208.32	9.97	168
Mo. longicollis	209.32	17.54	168
Mo. ramanniana var. ramanniana	112.08	11.74	192
Mo. parvispora	304.52	0.08	168
Genus : Mucor			
Mu. amphibiorum	763.74	32.99	48
Mu. ardhlaengiktus	210.8	11.52	52
Mu. azygosporus	292.63	13.18	168
Mu. bainieri	293.63	27.67	44
Mu. circinelloides f. griseocyanus	116.08	24.89	44
Mu. circinelloides f. circinelloides	119.08	30.90	44
Mu. circinelloides f. janssenii	232.29	24.32	44
Mu. circinelloides f. circinelloides	203.28	36.72	44
Mu. circinelloides f. lusitanicus	108.17	30.62	52
Mu. fragilis	236.35	24.95	52
Mu. fuscus	132.22	20.96	48
Mu. hiemalis f. hiemalis	110.19	24.33	48
Mu. minutus	586.67	26.35	52
Mu. mousanensis	999.7	23.40	52
Mu. oblongisporus	569.7	13.10	48
Mu. plumbeus	111.07	18.85	96
Mu. prayagensis	816.7	27.78	48
Mu. recurvus v. indicus	786.7	33.63	48
Mu. recurvus var. recurvus	317.52	7.09	44
Mu. rouxii	416.77	40.20	48
Mu. sinensis	204.74	0.73	52
Mu. subtilissmus	735.7	31.93	96
Mu. tuberculosporus	562.66	18.41	144
Mu. variabilis	564.66	33.26	44
Mu. variosporus	836.7	33.42	48
Mu. zychae var. zychae	416.67	17.71	72
Genus : Rhizomucor			
Rhizomucor miehei	182.67	11.53	48
Rhizomucor pusillus	354.68	4.50	96
Genus : Rhizopus			
Rhizopus homothallicus	336.62	19.88	192
Rhizopus microsporus var. chinensis	631.82	13.34	44
Rhizopus microsporus var. microspor.	699.68	11.77	48
Rhizopus microsporus var. rhizopodiformis	536.8	11.61	48
Rhizopus oryzae	112.07	10.27	44
Rhizopus stolonifer var. reflexus	319.35	16.33	96
Rhizopus stolonifer var. stolonifer	609.82	17.24	72

E X A M P L E 4

Preparation of Inoculant of Oleaginous Microorganisms

An alternative method to the one described in Example 2 for the preparation of an inoculant of the organisms referred to Example 3 was developed.

Sabouraud agar was placed in Roux flasks [or pencillin in flasks] and inoculated with the organism to be grown by washing the organism from cultures grown in petri dishes with 10 ml phosphate buffer. The wash was spread over the Sabouraud agar in the Roux flasks whereafter the flasks were incubated for 2 to 5 days at 30°C under forced, moist and sterile air introduced into the flasks. The resultant growth was washed from the flasks with phosphate buffer with 0,2% Tween 80 and used as inoculant.

E X A M P L E 5

Cultivation of M. javanicus on Acetic Acid as carbon source

To further demonstrate the ability of *Mucor circinelloides f. circinelloides* [CBS 203.28] [also referred to by the trivial name *M. javanicus*] to assimilate acetic acid as carbon source in the production of GLA a growth medium was prepared as follows:

H_2O	4 litres
Citric Acid	0,5g
NH_4Cl	3,2g
$MgSO_4.7H_2O$	1,6g
KH_2PO_4	6,0g
$CaCl_2.2H_2O$	0,1g
Yeast Extract	2,0g
Acetic Acid	8,0g

pH was set to 5,5 with KOH.

The following ingredients were added as trace elements:

$FeSO_4.7H_2O$	0,01g/L
$ZnSO_4.7H_2O$	0,01g/L
$MnSO_4.4H_2O$	0,001g/L
$CuSO_4.5H_2O$	0,0005g/L

4 Litres of the medium was placed in 5 litre fermenters and inoculated with 400 ml [i.e. 10%] inoculum prepared over a growth period of 24 hours in vortex aerated bottles stirred at 600 r.p.m. at 30°C. The composition of the inoculum medium was as follows:

H_2O	800 ml
Glucose	24g
KH_2PO_4	5,6g

Na_2HPO_4	1,6g
$MgSO_4.7H_2O$	1,2g
Yeast Extract	1,2g
$CaCl_2$	0,08g
Sodium glutamate	2,4g

All media were autoclaved.

The growth conditions in the 5 litre fermenter were as follows:

pH	5,5
Stirring rate	700 rpm
Temperature	30°C
Aeration	0,5 vvm
Antifoam	A small amount [3 drops] added at start of fermentation run

50% acetic acid was added to the medium from a supply reservoir to keep the pH at 5,5 during the growth period.

The acetic acid utilisation was determined according to the volume of acetic acid used from the supply reservoir and volume of culture in the fermenter.

The increase of biomass over time, the lipid content as a percentage of biomass and the gamma linolenic acid content of the fatty acids produced were determined. The results are set out in Table 2.

TABLE 2

Cultivation of Mucor javanicus with Acetic Acid

Time [hrs]	Biomass [gL^{-1}]	Resid. N_2 [NH_4^+]	Acetic acid utilised [ml/L]	Lipid % Biomass	% GLA of F.A.
0	0,1				
13	2,4	Depleted			
20	4,1	-	9,4	18,2	13,9
37	6,0	-	20,1	21,0	16,2
44	6,2	-	23,1	20,0	15.6
61	5,4	-	28,1	21,5	15,7
68	6,9	-	29,0	21,6	15,4
85	7,8	-	31,9	19,5	16,0

It is believed that the nutritional stress under which the organism was placed due to the fact that the nitrogen in the medium was depleted within 13 hours contributed to the high GLA production. By the process of the invention the acetic acid, which is toxic to the organisms at levels in excess of about 10-20g/l is slowly titrated into the culture medium such that the total acid content remains below toxic levels. It will be seen that by this procedure the organisms utilised a total of 31.9 ml/l acetic acid.

EXAMPLE 6

The performance of Mucor javanicus [i.e. Mucor circinelloides f. circinelloides] in the production of GLA from glucose as a carbon source material was compared with its performance when acetic acid is used as carbon source material. The strain used in this experiment was not the same as the one used in the experiment of Example 5 but is also available from the open collection of CBS in Baarn, Netherlands under number CBS 108.16.

A 5 litre fermenter was provided with 4 litres of a glucose-based growth medium of the following composition in water.

Glucose	50 gL^{-1}
$(NH_4)_2SO_4$	1,7gL^{-1}
KH_2PO_4	2,0gL^{-1}
$MgSO_4.7H_2O$	0,5gL^{-1}
$CaCl_2.2H_2O$	0,2gL^{-1}
$FeSO_4.7H_2O$	0,01gL^{-1}
$ZnSO_4.7H_2O$	0,01gL^{-1}
$MnSO_4.4H_2O$	0,001gL^{-1}
$CuSO_4.5H_2O$	0,0005gL^{-1}
Yeast Extract	1gL^{-1}

The pH of the medium was set at 5,5 with KOH.

400 ml of an inocolum containing Mucor javanicus strain CBS 108.16 in an inocolum medium of the same composition and prepared as described in Example 5 was used to inoculate the above growth medium. The growth conditions were also the same as described in Example 5.

In the comparative trial a 5 litre fermenter was provided with 4 litres of an acetic acid based growth medium of the following composition:

H_2O	4L
Citric Acid	0,5g
NH_4Cl	5,52g
$MgSO_4.7H_2O$	1,6g
KH_2PO_4	6,0g
$CaCl_2.2H_2O$	0,1g
Yeast Extract	4,0g
Acetic Acid	20,0g

Trace elements in the quantities listed in Example 5 were also added to the growth medium.

The medium was incoluated with 400 ml of the inoculum containing M. javanicus [CBS 108.16] as described above. The growth conditions were the same as for the glucose-based growth medium. However, throughout the growth period a solution of 50% [v/v] acetic acid in water was slowly titrated from a reservoir into the growth medium to maintain the pH of the growth medium at 5,5. In this manner it was ensured that the acetic acid content of the growth medium remained at a sub-lethal [i.e. non-toxic] level of 5g/l. The cultivation was carried on in this manner for a period of 162 hours and during this period the acetic acid utilisation, determined according to the volume of acetic acid used from the reservoir and the volume of culture in the fermenter, was determined to have been 81,4 ml/L which quantity would have been toxic to the organism if originally present in the fermenter.

Various determinations were made in comparing the performance of the microorganism on the two culture media. The results are set out in Table 3.

TABLE 3

Comparison of Performance of **M. javanicus** in GLUCOSE and ACETIC ACID

Parameters		Glucose 50g/L	Acetic Acid 5g/L
Total Lipid Content of mold [% w/w]		22,7 [72h]	22,6 [92h]
Total Neutral Lipid [NL] fraction [% w/w]		20,4 [72h]	18,1 [92h]
GLA % of total F.A. in NL-fraction		15,1 [72h]	13,9 [92h]
GLA content of mold [NL-fraction] [% w/w]		3,1	2,5
Biomass Density [g dw/L]	After 40h	12,0	11,4
	After 70h	16,6	14,4
	After 85h	16,5	15,4
Biomass Yield [gdw/source* util.]	After 40h	0,30	0,36†
	After 70h	0,33	0,29
	After 85h	0,33	0,28
GLA-Yield [gGLA/source* util.]	After 40h	0,010	0,007†
	After 70h	0,012	0,007
	After 85h	0,012	0,009
Production Time [h]		92	162
Lipid Yield [g lipid/ source util.]	After 40h	0,07	0,07†
	After 70h	0,08	0,06
	After 85h	0,07	0,06
Lipid Content [g/L] [% Lipid x 7]	After 40h	2,9	2,3†
	After 70h	3,8	3,0
	After 85h	3,6	3,4
GLA Content [g/L] [% GLA x 12]	After 40h	0,41	0,24†
	After 70h	0,57	0,33
	After 85h	0,57	0,45
GLA Content [mg/g dw] [13/7]	After 40h	34,2	19,2†
	After 70h	34,3	23,0
	After 85h	34,5	29,0

Notes:

* Total source utilised excludes ca. 12g glucose

from inoculum [12g glucose/400 ml medium added as 10% inoculum]. Values indicate glucose utilised [50g/L] in fermenter medium and ml acetic acid utilised for acetic acid based medium.

F.A. fatty acid

dw dry weight

† Samples taken after 44h

All data were read from graphs and are rounded off

The methods followed in the determinations set out above were as follows:

Dry weight determination. Culture biomass was determined by filtration of 2ml of culture through a pre-weighed membrane filter [GF/B, Whatman], washed with distilled water [6 x 2ml] and dried to a constant weight at 110°C. This procedure was performed in duplicate.

Lipid extraction. This was performed on freeze-dried material as described by Kendrick & Ratledge [1992] and include extraction with chloroform/methanol [2:1, v/v] as described by Folch et al. [1957], three washes with distilled water and final evaporation of the organic phase. Lipid material was finally dissolved in a minimal volume of diethyl ether and transferred to pre-weighed vials. For determination of lipid weights, samples were dried to constant weight in a vacuum oven at 50°C over P_2O_5.

Fractionation of extracted lipid. Extracted lipid was dissolved in chloroform and applied to a column [140 mm x 20 mm] of activated [by heating overnight at 110°C] silicic acid. Neutral-, sphingo- and glyco-lipids [as a combined fraction], as well as polar lipids, were eluted by successive applications of organic solvents as described by Kendrick & Ratledge [1992]. Final solvent removal and storage was as for whole lipid extracts. Each fraction was then purified further by thin layer chromatography.

Thin-layer chromatography. Thin-layer chromatography of the neutral-, sphingo- and glyco-lipid fraction was on silica gel thin-layer plates backed with aluminium [Merck]. The polar fraction, containing phospholipids, was separated using chloroform/methanol/water/acetic acid [65:43:3:1 by vol.].

The neutral lipid fraction was separated using petroleum ether [60 to 80°C]/diethyl ether/acetic acid [85:15:1, by vol.]. The combined sphingo- and glyco-lipid fraction was separated using chloroform/methanol/NH_4OH, sp. gr. 0,880, [80:20:0,2, by vol.]. Plates were developed one dimensionally by the ascending technique and visualised by exposure to I_2 vapour. Phospholipids were further visualised by staining with Dragendorff reagent, which specifically stains cholinecontaining lipids, ninhydrin for the detection of lipids with free amino groups [Higgins, 1987] and a molybdenum blue reagent spray for the detection of phospholipids in general by staining phosphate-containing lipids [Dittmer & Lester, 1964]. Glyco-lipids were visualised by spraying with alphanaphthol solution [Higgins, 1987]. Further identification was achieved by running suitable authentic standards alongside experimental samples.

Fatty acid analysis. Lipid was dissolved in chloroform and methylated by the addition of trimethyl sulphonium hydroxide [TMSH] as described by Butte [1983].

The FAME were analysed using a Phillips PU 4500 gas chromatograph and a 10% diethylene glycol succinate column [2 m 4 mm] as described by Kendrick & Ratledge [1992]. Identification of peaks was by reference to authentic standards.

Glucose analysis. Glucose was analysed by the GOD-PERID Test [Boehringer Mannheim].

Nitrogen analysis. Performed by estimating ammonia by the indophenol method [Chaney, A.L. and Marbach, E.P. (1962) Clinical Chemistry 8, 130].

From the results set out in Table 3 it will be seen that the performance of M. javanicus [CBS 108.16] on glucose was virtually the same as on acetic acid employed in the manner described herein. This is a most unexpected result.

The references referred to in the above examples are as follows:

Butte, W. (1983). Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxide for transesterification. **Journal of Chromatography** 261, 142-145.

Dittmer, J.C. & Lester, L. (1964). A simple specific spray for the detection of phospholipids on thin-layer chromatograms. **Journal of Lipid Research** 5, 126.

Folch, J., Lees, M. & Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. **Journal of Biological Chemistry** 226, 497-509.

Higgins, J.A. (1987). Separation and analysis of membrane lipid components, In **Biological membranes - a practical approach**, pp. 103-137. Edited by J.B.C. Findlay & W.H. Evans. Washington D.C.: IRL Press.

Kendrick, A.J. & Ratledge, C. (1992). Lipids of selected molds grown for production of n-3 and n-6 polyunsaturated fatty acids. **Lipids** 27, 15-20.

Kock, J.L.F. et. al. (1985). A rapid method to differentiate between four species of the Endomycetaceae. Short Comm. **J. Gen. Microbiol.** 131 : 3393-3396.

Many variations of the invention may be devised without departing from the spirit of the invention. Thus the fermentation process may be carried out in a continuous process.

CLAIMS

1. A method for producing a single cell oil containing gamma-linolenic acid characterised in that at least one microorganism of the order Mucorales is cultured in a growth medium which is substantially starch and sugar free and which contains, as a carbon source material, at least one monocarboxylic acid of 2 to 5 carbon atoms and recovering the oil from the resultant cultured microorganism biomass.

2. The method of claim 1 wherein the microorganism is of the family Mortierellaceae, preferably of the genus Mortierella and most preferably selected from the genera consisting of Mo. isabellina, Mo. longicollis and Mo. ramanniana var. ramanniana.

3. The method of claim 1 wherein the microorganism is of the family Mucoraceae.

4. The method of claim 3 wherein the microorganism is selected from the group consisting of the genera Actinomucor, Mucor, Rhizomucor and Rhizopus.

5. The method of claim 4 wherein the microorganism is selected from the group consisting of the following species of the genus Mucor, namely:

<u>Mu.</u> <u>amphibiorum</u>
<u>Mu.</u> <u>ardhlaengiktus</u>
<u>Mu.</u> <u>azygosporus</u>
<u>Mu.</u> <u>bainieri</u>
<u>Mu.</u> <u>circinelloides f. griseocyanus</u>
<u>Mu.</u> <u>circinelloides f. circinelloides</u>
<u>Mu.</u> <u>circinelloides f. janssenii</u>
<u>Mu.</u> <u>circinelloides f. lusitanicus</u>
<u>Mu.</u> <u>fragilis</u>
<u>Mu.</u> <u>fuscus</u>
<u>Mu.</u> <u>hiemalis f. hiemalis</u>
<u>Mu.</u> <u>minutus</u>
<u>Mu.</u> <u>mousanensis</u>
<u>Mu.</u> <u>oblongisporus</u>
<u>Mu.</u> <u>plumbeus</u>
<u>Mu.</u> <u>prayagensis</u>
<u>Mu.</u> <u>recurvus var. indicus</u>
<u>Mu.</u> <u>recurvus var. recurvus</u>
<u>Mu.</u> <u>rouxii</u>
<u>Mu.</u> <u>sinensis</u>
<u>Mu.</u> <u>subtilissmus</u>
<u>Mu.</u> <u>tuberculosporus</u>
<u>Mu.</u> <u>variabilis</u>
<u>Mu.</u> <u>variosporus</u>
<u>Mu.</u> <u>zychae var. zychae</u>

6. The method of any one of claims 1 to 5 wherein the growth medium comprises a mixture of acetic acid and assimilable sources of nitrogen and phosphate.

7. The method of claim 6 wherein the growth medium in addition also includes at least one of propanoic acid, butanoic acid, isobutyric acid, n-valeric acid and i-valeric acid and also assimilable sources of potassium and sulphur and trace elements required to sustain the growth of micro-organisms.

8. The method of claim 1 wherein the carbon source material is the aqueous organic acid stream

derived from a Fischer-Tropsch process.

9. The method of claim 1 wherein the total organic acid content of the culture medium is maintained at less than 10 g/l, more preferably between 1 and 5 g/l by addition of the acid on demand as the acid is utilised by the organisms.

10. A method of reducing the mono-carboxylic acid content of the aqueous organic acid stream of the Fischer-Tropsch process comprising the steps of cultivating a microorganism selected from the order Mucorales with the acid stream as growth medium and separating the biomass resulting from such cultivation from the medium.

11. The use of a C_2 to C_5 mono-carboxylic acid as primary carbon source in culturing microorganisms of the order Mucorales for the production of single cell oils.

12. An oil containing gamma-linolenic acid produced by the method of any one of claims 1 to 9.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/02288

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12P7/64; C10G2/00; C02F3/34

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12P ; C02F

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	BIOTECHNOLOGY LETTERS vol. 10, no. 5, 1988, pages 347 - 350 J. SAJBIDOR ET AL. 'Influence of different carbon sources on growth, lipid content and fatty acid composition in four strains belonging to mucorales' see the whole document	1-5,11
A	PATENT ABSTRACTS OF JAPAN vol. 11, no. 192 (C-429)(2639) 19 June 1987 & JP,A,62 14 791 (AGENCY OF IND. SCIENCE & TECHNOL.) 23 January 1987 see abstract	1,2,6,9

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 FEBRUARY 1993	15. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MONTERO LOPEZ B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	CHEMICAL ABSTRACTS, vol. 109, 1988, Columbus, Ohio, US; abstract no. 167036x, ZHELIFONOVA, V.P. ET AL. 'Lipid synthesis by Mucorales fungi growing in a medium with ethanol' page 402 ;column R ; see abstract & MIKROBIOLOGIYA vol. 57, no. 4, 1988,	12
A	---	1,3
A	WO,A,9 113 997 (BASF K&F CORPORATION) 19 September 1991 see page 3, line 21 - page 4, line 8 see page 5, line 19 - line 23 see page 7, line 4 - line 9 see page 8, line 20 - line 23 -----	1,3-7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9202288 SA 67518

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 23/02/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9113997	19-09-91	AU-A- 7567091	10-10-91
		EP-A- 0520027	30-12-92

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82