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PROCESS FOR CULTIVATING MICROORGANISMS ON A HYDROCARBON FEEDSTOCK EMPLOYING A CARBOHYDRATE PRETREATMENT FEEDSTOCK

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This invention relates to the cultivation of yeast by a process comprising starting the growth of yeast on a carbohydrate starter feedstock followed by growth on a main feedstock containing a straight chain hydrocarbon.

According to the present invention there is provided a process which comprises, in a pretreatment stage, cultivating a microorganism in the presence of a pretreatment feedstock comprising a compound containing carbon, hydrogen and oxygen and thereafter, in a growth stage, cultivating the microorganism product so obtained in the presence of a growth feedstock comprising a straight chain hydrocarbon.

By operating in this manner the lag phase, which usually occurs when a microorganism is first cultivated on a hydrocarbon feedstock, is reduced in its effect or is eliminated.

It will be necessary, in both the pretreatment stage and in the growth stage to operate in the presence of a gas containing free oxygen and in the presence of an aqueous nutrient medium. This medium may be of different composition in the two stages.

When operating in batchwise manner the two stages may be carried out in either the same or different fermenters.

When operating in continuous manner separate fermenters or separate zones in the same fermenter will be necessary.

Usually the feedstock used in the pretreatment stage will be a carbohydrate, for example consisting of or contained in molasses, wort, malt extract, wood hydrolysis sugars or lyes obtained in the course of paper manufacture. A special pretreatment feedstock which may be employed is an extract of lipids obtained in the purification of yeasts by solvent extraction.

Preferably the hydrocarbon is C_{10} or higher. Suitably there may be used a hydrocarbon fraction derived from petroleum.

It is well-known that certain petroleum fractions, particularly gas oils, contain straight chain hydrocarbons, mainly paraffins which are waxes and which have an adverse effect upon the pour point of the fraction; that is to say, when these hydrocarbons are removed, wholly or in part, the pour point of the fraction is lowered. Usually the wax is removed by precipitation by means of solvents, the wax originally present in the fraction being recovered as such, that is, without conversion to more valuable products.

The petroleum fractions boiling below the gas oils, for example, heavy naphthenes and kerosines also contain straight chain hydrocarbons which are potentially valuable for conversion to other products but hitherto, in general, utilisation of these hydrocarbons has been rendered difficult by the necessity of recovering these hydrocarbons from the petroleum fractions, in which they are contained, before they can be converted to other products.

According to a preferred feature of this invention there is provided a process which comprises cultivating a microorganism in the manner as hereinbefore described in the presence of a petroleum fraction consisting in part of

straight chain hydrocarbons and having a mean molecular weight corresponding to at least 10 carbon atoms per molecule, and in the presence of an aqueous nutrient medium; and in the presence of a gas containing free oxygen and separating from the mixture, on the one hand, the microorganism and, on the other hand, a petroleum fraction having a reduced proportion of straight chain hydrocarbons or which is free of said straight chain hydrocarbons.

The process of the invention is of particular value for the treatment of petroleum gas oil fractions which contain straight chain hydrocarbons in the form of waxes, since by the process of the invention, a gas oil of improved pour point is obtained while the waxes are converted to a valuable product.

Usually the straight-chain hydrocarbons will be present in the feedstocks according to the invention as paraffins; however, the straight chain hydrocarbons may be present as olefins; also there may be used a mixture containing straight chain paraffins and olefins.

It is an important feature of this invention that when cultivating yeasts in the presence of the feedstock hereinbefore described under conditions favouring the growth of the yeasts at the expense of the straight chain hydrocarbons, the other hydrocarbons, for example isoparaffins, naphthenes and aromatics are not metabolised or, at most, the proportion which is metabolised is very small. Furthermore, unlike conventional chemical processes governed by the law of mass action, the rate of removal of straight chain hydrocarbons is not substantially reduced as the proportion of these hydrocarbons in the overall mixture of hydrocarbons decreases (except, of course, in the very final stages of removal). Thus, when desired, the percentage conversion of straight chain hydrocarbons which is achieved can be maintained at a value approaching 100% without necessitating a very disproportionate expenditure of contact time to achieve small improvements. Furthermore, in the continuous process, this high percentage conversion can be achieved without resorting to the use of a long reaction path.

By the application of this process under conditions which limit the metabolisation of the straight chain hydrocarbons it is possible to operate with the removal of only a desired proportion of these hydrocarbons.

Suitable feedstocks to the process of the invention include kerosine, gas oils and lubricating oils; these feedstocks may be unrefined or may have undergone some refinery treatment, but will usually be required to contain a proportion of straight chain hydrocarbons in order to fulfill the purpose of this invention. Suitably the petroleum fraction will contain 3–45% by weight of straight chain hydrocarbons.

Microorganisms which are cultivated as herein described may be yeasts, moulds or bacteria.

Preferably when a yeast is employed this is of the family Cryptococcaceae and particularly of the sub-family Cryptococcoideae; however, if desired there may be use, for example, ascosporogeneous yeasts of the sub-family Saccharomycoideae. Preferred genera of the Cryptococcoideae sub-family are Torulopsis (also known as Torula) and Candida. Preferred strains of yeast are as follows. In particular it is preferred to use the specific stock of indicated Baarn reference numbers; these reference numbers refer to a stock held by the Centraal Bureau vor Schimmelculture, Baarn, Holland:

Candida lipolytica
Candida pulcherirma CBS 610
Candida utilis
Candida utilis, var. *major* CBS 841
Candida tropicalis CBS 2317
Torulopsis collisculosa CBS 133

Hansenula anomala CBS 110
Oidium lactis
Neurospora sitophila
Mycoderma cancoillote

Of the above *Candida lipolytica* is particularly preferred.

If desired, the microorganism may be a mould. A suitable strain is *Penicillium expansum*.

If desired, the micro-organism may be a bacterium. Suitably the bacteria are of one of the orders: Pseudomonadales, Eubacteriales and Actinomycetales.

Preferably the bacteria which are employed are of the family Bacillaceae and Pseudomonadaceae. Preferred species are *Bacillus megaterium, Bacillus subtilis* and *Pseudomonas aeruginosa*. Other strains which may be employed include:

Bacillus amylebacter
Pseudomonas natriegens
Arthrobacter sp.
Micrococcus sp.
Corynebacterium sp.
Pseudomonas syringae
Xanthomonas begoniae
Flavobacterium devorans
Acetobacter sp.
Actinomyces sp.

Suitable moulds are of the family Aspergillaceae. A suitable genus is Penicillium.

Preferably there is used *Penicillium expansum*. Another suitable genus is Aspergillus.

Usually the cultivation is carried out in the presence of an aqueous nutrient medium. If desired, certain solid nutrient media may be employed.

In either case, a gas containing free oxygen must be provided.

Penicillium expansum is suitable for cultivation in an aqueous nutrient medium containing hydrocarbons.

Penicillium roqueforti, Penicillium notatum, Aspergillus fussigatus and *Aspergillus niger, Aspergillus versicolor* may be used for cultivation on a solid agent containing hydrocarbons as feedstock.

For the growth of the microorganism it will be necessary to provide, in addition to the feedstock, an aqueous nutrient medium and a supply of oxygen, preferably in the form of air.

A typical nutrient medium for the growth of Nocardia, a genus in the Actinomycetales order, has the following composition:

	Grams
Ammonium sulphate	1
Magnesium sulphate	0.20
Ferrous sulphate, $7H_2O$	0.005
Manganese sulphate, $1H_2O$	0.002
Monopotassium phosphate	2
Disodium phosphate	3
Calcium Chloride	0.1
Sodium Carbonate	0.1
Yeast Extract	0.008

Distilled water (to make up to 1000 mls.).

For other bacteria a suitable nutrient medium has the composition:

Monopotassium phosphate	gm.	7
Magnesium sulphate, $7H_2O$	gm.	0.2
Sodium chloride	gm.	0.1
Ammonium chloride	gm.	2.0
Tap water (trace elements)	ml.	100
Yeast extract	gm.	0.025

Made up to 1000 mls. with distilled water.

A suitable nutrient medium for yeasts (and moulds) has the composition:

	Grams
Diammonium phosphate	2
Potassium chloride	1.15
Magnesium sulphate, $7H_2O$	0.65
Zinc sulphate	0.17
Manganese sulphate, $1H_2O$	0.045
Ferrous sulphate, $7H_2O$	0.068
Tap water	200
Yeast extract	0.025

Distilled water (to make up to 1000 mls.).

The growth of the microorganism used is favoured by the addition to the culture medium of a very small proportion of extract of yeast (an industrial product rich in vitamins of group B obtained by the hydrolysis of a yeast) or more generally of vitamins of group B and/or biotin. This quantity is preferably of the order of 25 parts per million with reference to the aqueous fermentation medium. It can be higher or lower according to the conditions chosen for the growth.

The growth of the microorganism takes place at the expense of the feedstock fraction with the intermediate production of bodies having an acid function, principally fatty acids, in such manner that the pH of the aqueous mineral medium progressively diminishes. If one does not correct it the growth is fairly rapidly arrested and the concentration of the microorganism in the medium, that is cellular density, no longer increases so that there is reached a so-called stationary phase.

Preferably therefore the aqueous nutrient medium is maintained at a desired pH by the step-wise or continuous addition of an aqueous medium of high pH value. Usually, when using moulds or yeasts and in particular when using *Candida lipolytica*, the pH of the nutrient medium will be maintained in the range 3–6 and preferably in the range 4–5. (Bacteria require a higher pH, usually 6.5–8.) Suitable alkaline materials for addition to the growth mixture include sodium hydroxide, potassium hydroxide, disodium hydrogen phosphate and ammonia, either free or in aqueous solution.

The optimum temperature of the growth mixture will vary according to the type of microorganism employed and will usually lie in the range 25–35° C. When using *Candida lipolytica* the preferred temperature range is 28–32° C.

The take-up of oxygen is essential for the growth of the microorganism. The oxygen will usually be provided as air. In order to maintain a rapid rate of growth the air, used to provide oxygen, should be present in the form of fine bubbles under the action of stirring. The air may be introduced through a sintered surface. However there may be used the system of intimate aeration known as "vortex aeration."

It has been found that by the use of yeast of the strain *Candida lipolytica* in a process according to the invention in which aeration is effected by "vortex aeration," a high growth rate is achieved whereby the generation time lies in the range 2–5 hours and the cell concentration is increased by a factor of up to 12 in two days.

In batch operation, the microorganism will usually grow initially at a low rate of increase in cellular density. (This period of growth is referred to as the "lag phase".) Subsequently the rate of growth will increase to a higher rate of growth; the period at the higher rate of growth is referred to as the "exponential phase" and subsequently again the cellular density will become constant (the "stationary phase").

A supply of the microorganism for starting the next batch will preferably be removed before the termination of the exponential phase.

The growth operation will usually be discontinued before the stationary phase.

At this stage, the microorganism will usually be sepa-

rated from the bulk of the aqueous nutrient medium and from the bulk of the un-used feedstock fraction.

If desired the microorganism may be subjected to autolysis before further purification of the product.

According to one method of treating the product the major part of the continuous aqueous phase is first separated; preferably this is carried out by centrifuging, or decanting. The separated aqueous phase will usually contain a greater concentration of non-nutritive ions than can be tolerated in the recycle stream and when this is so, only a proportion of the recovered aqueous phase can be recycled. Thus it will usually be possible to separate ca. 96% by wt. of the aqueous phase which is present in the product, of which on the same percentage basis, ca. 20% by wt. will be discarded. The recycle stream is supplied with make-up quantities of the necessary nutrients and is returned to the fermenter; if desired the make-up materials may be fed to the fermenter as a separate stream.

The process, as applied to the cultivation of a yeast, may incorporate product separation stages as follows. In some cases microorganisms other than yeasts may be separated in this manner.

By centrifuging the product from the fermenter three fractions are recovered. These are in order of increasing density:

(i) an oil phase containing yeast cells
(ii) an aqueous phase containing traces of oil and yeast, and
(iii) a yeast "cream" consisting of yeast, having a quantity of oil fixed onto the cells, together with aqueous phase.

After recovery of fraction (ii), fraction (iii) or a blend of fractions (i) and (iii) is mixed with an aqueous solution of a surfactant.

The purpose of this treatment is to separate at least part of the oil from the yeast cells; the oil being apparently held to the cells by adsorption.

It may be advantageous to employ an edible surfactant, for example a saccharose ester, which makes it possible to reduce the subsequent washing required to remove from the yeast a surfactant which is not edible.

The emulsion so formed is broken down by centrifuging to obtain three fractions:

(iv) an oil phase
(v) an aqueous phase containing surfactant, which phase is recycled for the treatment of fractions (i) and (iii), and
(vi) a yeast "cream," consisting of yeast still contaminated by oil together with an aqueous surfactant phase.

In order to reduce as far as possible the consumption of surfactant product, the aqueous washing solution containing it is recycled.

Fraction (vi) may be further treated by alternate washing with surfactant and centrifuging until the oil content of the yeast has reached a desired low value. The yeast "cream" now consisting of yeast and aqueous surfactant may now be washed with water and again centrifuged. If desired two or more washings may be given to this yeast "cream." If desired, one or more of these water washings (but preferably not the last) may make use of salt water (for example sea water); preferably the final wash is with soft water. With a view to economising the soft water necessary for the process, the whole of this water coming from the last washing is employed for making up the nutritive medium for the fermentation, where necessary at the stage of washing with the solution of surfactant, and the rest is sent to the salt water used for washing with a view to reducing its salt concentration. Finally the yeast may be dried under conditions suitable for its subsequent use as a foodstuff.

Other steps which may be taken to obtain a purified microorganism or a product derived therefrom or to improve the process in respect of the production of the unmetabolised hydrocarbon fraction are described in the following applications; the use of any process step or steps therein described in association with the process herein described lies within the scope of the present invention.

The stages of the process may be carried out entirely batchwise. However, if desired, any one or more stages herein described may be carried out in continuous manner.

The invention is illustrated but not limited with reference to the following experiment and example.

Throughout these examples cellular density is expressed as dry weight of yeast per litre of culture.

The experiment is provided for purposes of comparison and does not constitute operation according to the present invention.

Experiment

40 litres of an aqueous mineral nutrient medium were introduced into a stainless steel fermenter having an effective capacity of 60 litres.

20 litres of an inoculum of 24 hr. *Candida lipolytica* culture on normal paraffinic hydrocarbons (in the stationary growth phase, cellular density 5 grams/litre) was then introduced into the fermenter, giving a cellular density of about 1 gram/litre in the fermenter.

1.03 litres, that is 15 grams/litre, of heavy gas-oil was then added to the fermenter, that is sufficient to take the cellular density to 2 grams/litre.

The temperature of the culture was regulated at 30±1° C., pH 4, aeration and agitation to give 3 millimoles of O_2 per litre of medium per minute. An automatic pH controller added 10 N ammonia as required.

When the flow of ammonia reached 20 ml. the addition of gas-oil was started following the theoretical needs of the culture, assuming a yield

$$\left(100 \times \frac{\text{dry yeast}}{\text{gas-oil required}}\right)$$

of 10% and a cell division time of 3 hours. This addition was carried out every hour until a total of 250 grams/litre of gas-oil had been added, i.e. 17 litres.

The time taken for this addition was 15 hours. Starting with a density of 1 gram/litre a lag period of 5 hours followed; then an acceleration phase of 3 hours and finally a phase of exponential growth which continues to 18 grams/litre at 22 hours.

Example

The method described in the experiment was repeated but using, an inoculum, 20 litres of a 24 hr. *Candida lipolytica* culture on molasses (cellular density 6 grams/litre in the stationary growth phase). The inoculum was prepared using 30 grams/litre of beet molasses, containing among other growth factors 0.04 mg./kg. of biotin, 50 mg./kg. of pantothenic acid and 5 mg./kg. of inositol, these three factors being particularly valuable for the growth of yeasts. When the inoculum was employed on the heavy gas-oil there was no lag phase, the culture starting off immediately in the phase of maximum growth (cell division time 3 hours).

The aqueous nutrient medium used in the process of the experiment and example had the following composition:

	Grams
Diammonium phosphate	2
Potassium chloride	1.15
Magnesium sulphate, $7H_2O$	0.65
Zinc sulphate	0.17
Manganese sulphate, $1H_2O$	0.045
Ferrous sulphate, $7H_2O$	0.068
Yeast extract	0.025
Tap water	200

Distilled water to 1000 ml.

We claim:

1. A process which comprises, in a pretreatment stage, cultivating a carbohydrate consuming yeast in the presence of a pretreatment feedstock comprising a carbohydrate and thereafter, in a growth stage, cultivating the

yeast so obtained in the presence of a growth feedstock comprising a straight chain hydrocarbon.

2. A process according to claim 1 in which the carbohydrate is contained in a class of material selected from the group consisting of molasses, wort, malt extract, wood hydrolysis sugars and lyes obtained in the course of paper manufacture.

3. A process according to claim 1 in which the yeast is of the family Cryptococcaceae.

4. A process according to claim 3 in which the yeast is of the sub-family Cryptococcoideae.

5. A process according to claim 4 in which the yeast is of the genus Torulopsis.

6. A process according to claim 4 in which the yeast is of the genus Candida.

7. A process according to claim 4 in which the yeast is *Candida lipolytica.*

8. A process according to claim 1 in which the feedstock is a petroleum fraction.

9. A process according to claim 1 in which straight chain hydrocarbons are removed from a petroleum fraction with production of a yeast, wherein the feedstock employed for the growth of the micro-organism is a petroleum fraction consisting in part of straight chain hydrocarbons, whereby there is recovered from the product of the growth of the yeast a petroleum fraction having a reduced proportion of straight chain hydrocarbons or which is free of said straight chain hydrocarbons.

10. A process according to claim 9 for the removal, at least in part, of waxes from a wax-containing petroleum gas oil, wherein the feedstock is a wax-containing petroleum gas oil and wherein there is recovered, from the product of the growth of the microorganism, a gas oil of reduced content of wax.

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