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(54) Title: MPG ADDED TO FERMENTATION

(57) Abstract: A method for fermenting a microorganism, producing a polypeptide of interest, in a culture medium of at least 50 litres, comprising:adding one or more compounds selected from the group consisting of monopropylene glycol, ethylene glycol, trehalose, xylitol, arabitol, dulcitol, mannitol, erythritol, cellobiose and sorbitol, to the culture medium before and/or during fermentation, wherein the compound is low metabolizable.

MPG ADDED TO FERMENTATION

TECHNICAL FIELD

The present invention relates to a method of increasing solubility of a polypeptide of interest during fermentation.

BACKGROUND ART

Formation of polypeptide crystals/amorphous precipitate during fermentation is today seen frequently because the fermentation yields are getting higher and higher due to optimization of the fermentation recipes and/or due to identification/development or construction of more efficient production organisms.

In such cases, the polypeptides are fermented in yields that are above their solubility limit, meaning that they may be present in the culture broth in a partly precipitated form. The precipitate may be in the form of crystals or as amorphous precipitates.

This causes problems in recovery where special measures have to be taken to solubilize the crystals/amorphous precipitate before removing the cells and other solids from the culture broth. These measures often result in yield losses.

The purpose of this invention is therefore to provide a simple and efficient solution to the above described problem.

SUMMARY OF THE INVENTION

It has surprisingly been found that the polypeptide of interest can be prevented from crystallizing or precipitating by adding a carbohydrate and/or a polyol and/or a derivative thereof and/or a polymer to the culture medium before and/or during fermentation, wherein the microorganism is not, or only to a low extent, able to metabolize said carbohydrate and/or said polyol and/or said derivative thereof; in particular the present invention deals with:

A method for fermenting a microorganism, producing a polypeptide of interest, in a culture medium of at least 50 litres, comprising:

adding one or more compounds selected from the group consisting of 1,2-propandiol, 1,3-propandiol, ethylene glycol, trehalose, xylitol, arabitol, dulcitol, mannitol, erythritol, cellobiose, sorbitol and a polyether having an average molecular weight less than 1000, to the culture medium before and/or during fermentation, wherein the compound is low metabolizable measured by $(OD_{III}-OD_{II})/(OD_{I}-OD_{II}) < 25\%$ as defined herein.

DETAILED DISCLOSURE OF THE INVENTION

The present invention deals with a new and surprisingly effective way of preventing the polypeptide of interest to crystallize or precipitate during the fermentation.

We have surprisingly found that if small amounts of, e.g., 5 % w/w of monopropylene glycol (MPG) is present during the fermentation, the formation of crystals or amorphous precipitate can be avoided, significantly delayed or significantly reduced. The MPG is only a very poor carbon source for most microorganisms or is very poorly metabolized by most microorganisms, or not metabolized at all, so it can be added before starting the fermentation and/or added during the fermentation without affecting the cell growth and productivity of the peptide of interest significantly.

By avoiding formation of polypeptide crystals/amorphous precipitate during fermentation, a much more simple recovery process can be used resulting in higher yields.

Microorganisms

The microorganism (the microbial strain) according to the invention may be obtained from microorganisms of any genus.

In a preferred embodiment, the polypeptide of interest may be obtained from a bacterial or a fungal source.

For example, the polypeptide of interest may be obtained from a gram positive bacterium such as a *Bacillus* strain, *e.g.*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis*; or a *Streptomyces* strain, *e.g.*, *Streptomyces lividans* or *Streptomyces murinus*; or from a gram negative bacterium, *e.g.*, *E. coli* or *Pseudomonas* sp.

The polypeptide of interest may be obtained from a fungal source, e.g. from a yeast strain such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* strain, e.g., *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* strain.

The polypeptide of interest may be obtained from a filamentous fungal strain such as an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* strain, in particular the polypeptide of interest may be obtained from an *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*,

Aspergillus japonicus, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* strain.

Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide of interest is produced by the source or by a cell in which a gene from the source has been inserted.

Modification of the microorganism of interest

The microorganism used according to the present invention may be modified in such a way that it is not, or only to a low extent, able to metabolize the chosen carbohydrate and/or polyol and/or derivative thereof; e.g., the original microorganism is able to metabolize glycerol or cyclodextrin but the modified microorganism is not, or only to a low extent.

Polypeptide of interest

The polypeptide of interest may be a peptide or a protein.

A preferred peptide according to this invention contains from 2 to 100 amino acids; preferably from 10 to 80 amino acids; more preferably from 15 to 60 amino acids; even more preferably from 15 to 40 amino acids.

In a preferred embodiment, the protein is an enzyme, in particular a hydrolase (class EC 3 according to Enzyme Nomenclature; Recommendations of the Nomenclature Committee of the International Union of Biochemistry).

In a particular preferred embodiment the following hydrolases are preferred:

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are

included. The protease may be an acid protease, a serine protease or a metallo protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Preferred commercially available protease enzymes include ALCALASE™, SAVINASE™, PRIMASE™, DURALASE™, ESPERASE™, RELASE™ and KANNASE™ (Novozymes A/S), MAXATASE™, MAXACAL™, MAXAPEM™, PROPERASE™, PURAFECT™, PURAFECT OXP™, FN2™, and FN3™ (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g. from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g. from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas sp.* strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g. from *B. subtilis* (Dartois et al. (1993), Biochemica et Biophysica Acta, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include LIPOLASE™, LIPOLASE ULTRA™ and LIPEX™ (Novozymes A/S).

Amylases: Suitable amylases (alpha and/or beta) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, alpha-amylases obtained from *Bacillus*, e.g. a special strain of *B. licheniformis*, described in more detail in GB 1,296,839.

Examples of useful amylases are the variants described in WO 94/02597, WO 94/18314, WO 96/23873, WO 97/43424, and WO 01/66712, especially the variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154,

156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444.

Commercially available amylases are DURAMYL™, TERMAMYL™, FUNGAMYL™, NATALASE™, TERMAMYL LC™, TERMAMYL SC™, LIQUIZYME-X™ and BAN™ (Novozymes A/S), RAPIDASE™ and PURASTAR™ (from Genencor International Inc.).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus, Pseudomonas, Humicola, Fusarium, Thielavia, Acremonium,* e.g. the fungal cellulases produced from *Humicola insolens, Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

Commercially available cellulases include CELLUZYME™, CAREZYME™ , and CAREZYME CORE™ (Novozymes A/S), CLAZINASE™, and PURADAX HA™ (Genencor International Inc.), and KAC-500(B)™ (Kao Corporation).

Oxidoreductases

Oxidoreductases that may be treated according to the invention include peroxidases, and oxidases such as laccases, and catalases.

Other preferred hydrolases are carbohydrolases including MANNAWAY™. Other preferred enzymes are transferases, lyases, isomerases, and ligases.

Fermentations

The present invention may be useful for any fermentation in industrial scale, e.g. for any fermentation having culture media of at least 50 litres, preferably at least 100 litres, more preferably at least 500 litres, even more preferably at least 1000 litres, in particular at least 5000 litres.

The microbial strain may be fermented by any method known in the art. The fermentation medium may be a complex medium comprising complex nitrogen and/or carbon sources, such as soybean meal, soy protein, soy protein hydrolysate, cotton seed meal, corn steep liquor, yeast extract, casein, casein hydrolysate, potato protein, potato

protein hydrolysate, molasses, and the like. The fermentation medium may be a chemically defined media, e.g. as defined in WO 98/37179.

The fermentation may be performed as a batch, a fed-batch, a repeated fed-batch or a continuous fermentation process.

In a fed-batch process, either none or part of the compounds comprising one or more of the structural and/or catalytic elements is added to the medium before the start of the fermentation and either all or the remaining part, respectively, of the compounds comprising one or more of the structural and/or catalytic elements is fed during the fermentation process. The compounds which are selected for feeding can be fed together or separate from each other to the fermentation process.

In a repeated fed-batch or a continuous fermentation process, the complete start medium is additionally fed during fermentation. The start medium can be fed together with or separate from the structural element feed(s). In a repeated fed-batch process, part of the fermentation broth comprising the biomass is removed at time intervals, whereas in a continuous process, the removal of part of the fermentation broth occurs continuously. The fermentation process is thereby replenished with a portion of fresh medium corresponding to the amount of withdrawn fermentation broth.

In a preferred embodiment of the invention, a fed-batch, a repeated fed-batch process or a continuous fermentation process is preferred.

Carbohydrates

Slowly metabolizable carbohydrates such as pullulan, limit dextrin, and trehalose may be used according to the present invention.

In a particular embodiment of the invention the slowly metabolizable carbohydrate is added to the culture medium either prior to inoculation or after inoculation at an amount of at least 0.1 % (w/w); in particular at an amount of at least 0.5% (w/w). The slowly metabolizable carbohydrate is added to the culture medium either prior to inoculation or after inoculation at an amount of up to 10% w/w; preferably at an amount of up to 8% w/w; more preferably at an amount of up to 6% w/w; more preferably at an amount of up to 5% w/w; more preferably at an amount of up to 4% w/w; more preferably at an amount of up to 3% w/w; more preferably at an amount of up to 2% w/w; even more preferably at an amount of up to 1% w/w.

Polyols

A very useful subgroup of carbohydrates, polyols, may be used according to the

invention. Any polyol may be used. However, a polyol selected from the group consisting of 1,2-propandiol (monopropylene glycol), 1,3-propandiol, glycerol, ethylene glycol, xylitol, arabitol, dulcitol, mannitol, erythritol, cellobiose and sorbitol, is preferred.

It is to be noted that some polyols, e.g. glycerol, are rather easily metabolized by most cells, but the uptake of e.g. glycerol can be blocked, meaning that glycerol may be used according to the present invention.

In a particular embodiment of the invention the polyol is added to the culture medium either prior to inoculation or after inoculation at an amount of at least 0.1 % (w/w); in particular at an amount of at least 0.5% (w/w). The polyol is added to the culture medium either prior to inoculation or after inoculation at an amount of up to 10% w/w; preferably at an amount of up to 8% w/w; more preferably at an amount of up to 6% w/w; more preferably at an amount of up to 5% w/w; more preferably at an amount of up to 4% w/w; more preferably at an amount of up to 3% w/w; more preferably at an amount of up to 2% w/w; even more preferably at an amount of up to 1% w/w.

In some cases it may be an advantage to use a mixture of two or more polyols, e.g. glycerol and monopropylene glycol, or a mixture of a polyol and a slowly metabolizable carbohydrate.

Derivatives

Another very useful subgroup of carbohydrates, derivatives, may be used according to the invention. Derivatives that may be used include maillard products, methyl glycosides, glucoronic acids, amino sugars, or N-acetyl glucosamines.

In a particular embodiment of the invention the derivative is added to the culture medium either prior to inoculation or after inoculation at an amount of at least 0.1 % (w/w); in particular at an amount of at least 0.5% (w/w). The derivative is added to the culture medium either prior to inoculation or after inoculation at an amount of up to 10% w/w; preferably at an amount of up to 8% w/w; more preferably at an amount of up to 6% w/w; more preferably at an amount of up to 5% w/w; more preferably at an amount of up to 4% w/w; more preferably at an amount of up to 3% w/w; more preferably at an amount of up to 2% w/w; even more preferably at an amount of up to 1% w/w.

Polymers

Polymers such as polyethers having an average molecular weight less than 1000; preferably an average molecular weight less than 900; more preferably an average molecular weight less than 800; even more preferably an average molecular weight less than 700, (e.g.

polyethylene glycol 200 (PEG 200), polyethylene glycol 400 (PEG 400)), or their derivatives including block polymers or block copolymers of polyethylene oxide and polypropylene oxide, wherein the ends of the polymers may further be protected by an acyl group or an alkyl group, may also be used according to the present invention.

In a particular embodiment of the invention the polymer is added to the culture medium either prior to inoculation or after inoculation at an amount of at least 0.1 % (w/w); in particular at an amount of at least 0.5% (w/w). The polymer is added to the culture medium either prior to inoculation or after inoculation at an amount of up to 10% w/w; preferably at an amount of up to 8% w/w; more preferably at an amount of up to 6% w/w; more preferably at an amount of up to 5% w/w; more preferably at an amount of up to 4% w/w; more preferably at an amount of up to 3% w/w; more preferably at an amount of up to 2% w/w; even more preferably at an amount of up to 1% w/w.

Salts

In addition to adding a slowly metabolizable carbohydrate it may also be an advantage to add a salt to the fermentation medium (see e.g. Example 4).

A preferred salt is selected from the group consisting of a chloride, a sulphate, a phosphate, a nitrate, and an ammonium salt; e.g. NaCl, KCl, Na2SO4, K2SO4.

Extent of metabolization

The following test may be used to check whether a microorganism, producing a polypeptide of interest, is not, or only to a low extent, able to metabolize a given compound:

A suitable media for the growth of the microorganism of interest is chosen.
The media is characterized by the following parameters:
a: The media contains glucose as the only carbohydrate source.
b. When glucose is removed the media should only be able to support growth of a significantly lower biomass (less than 50%).

The growth of the microorganism of interest is then compared in the following 3 media:
I: Normal media (with glucose as the only carbohydrate source)
II: Media I without glucose
III: Media I without glucose, but with the same C-mol of the compound to be tested.

The growth is then followed for a period of 8 hr in the 3 above mentioned media. Inoculation

is done with a concentration of biomass that will secure that the normal media is outgrown in 75% of the time frame. The amount of biomass is measured as optical density (OD) at 650 nm. OD obtained in the different media is measured.

The compound to be tested is defined as low metabolizable, if

$(OD_{III}-OD_{II})/(OD_{I}-OD_{II}) < 25\%$; preferably

$(OD_{III}-OD_{II})/(OD_{I}-OD_{II}) < 20\%$; more preferably

$(OD_{III}-OD_{II})/(OD_{I}-OD_{II}) < 15\%$; more preferably

$(OD_{III}-OD_{II})/(OD_{I}-OD_{II}) < 10\%$; more preferably

$(OD_{III}-OD_{II})/(OD_{I}-OD_{II}) < 5\%$; more preferably

$(OD_{III}-OD_{II})/(OD_{I}-OD_{II}) = 0\%$

In Example 1 various compounds are tested according to this test.

Recovery of the polypeptide of interest

A further aspect of the invention concerns the downstream processing of the fermentation broth. After the fermentation process is ended, the polypeptide of interest may be recovered from the fermentation broth, using standard technology developed for the polypeptide of interest. The relevant downstream processing technology to be applied depends on the nature of the polypeptide of interest.

A process for the recovery of a polypeptide of interest from a fermentation broth will typically (but is not limited to) involve some or all of the following steps:

1) pre-treatment of broth (e.g. flocculation)
2) removal of cells and other solid material from broth (primary separation)
3) filtration
4) concentration
5) filtration
6) stabilization and standardization.

Apart from the unit operations listed above, a number of other recovery procedures and steps may be applied, e.g., pH-adjustments, variation in temperature, crystallization, treatment of the solution comprising the polypeptide of interest with active carbon, and use of various adsorbents.

By using the method of the invention the yield of the polypeptide of interest is much higher in the recovery when the crystal formation is reduced or eliminated by adding of , e.g. MPG, during fermentation.

The invention is further illustrated in the following examples, which are not intended

to be in any way limiting to the scope of the invention as claimed.

Example 1

Evaluation of the suitability of different polyols and carbohydrates as carbon-sources for micro-organisms.

Shake flask media:

Med-F 18 shake flask medium (concentrations are after final mixing).

Part A: Bacto-peptone 0.5; Yeast Extract 0.5 g/l; Magnesium sulphate, 7H2O 0.5 g/l; Ammonium sulphate 2 g/l; Calcium chloride, 2H2O 0.1 g/l; Citric acid 50 mg/l; trace metals (MnSO4, H2O 2.5 mg/l; FeSO4, 7H2O 9.9 mg/l; CuSO4, 5H2O 1.0 mg/l; ZnCl2 1.0 mg/l); PLURONIC™ 0.1 g/l; pH adjusted to 6.7.

Part B: 5 g/l Potassiumdihydrogenphosphate pH adjusted to 6.7 with NaOH.

Part C: carbon source equivalent to 0.08 mol carbon per liter (e.g. 2.5 g/l glucose)

Demineralized water is used for the preparation of all media.

After sterilization for 20 minutes at 121°C part A, B and C are mixed.

Strain: *Bacillus licheniformis*

Procedure for shake flask evaluation of suitability of different polyols and carbohydrates as alternative carbon-sources:

First the cells were grown in a pre-culture that secured good growing cells.

Each shake flask is then inoculated with the same amount of cells based on the OD (650 nm) measurement.

An inoculum strength of OD x ml cell suspension = 80 was used in this case. Resulting in an OD=0.8 in the shake flask at time 0.

Three shake flasks of each type were inoculated.

Shake flask types:

I: Med-F 18 with the normal part C added, where C is glucose (resulting in a medium with 2.5 g/l of glucose (equivalent to 0.08 C-mol per Liter).

II: Med-F 18 without part C, resulting in a medium without any glucose

III: Med-F 18 with part C replaced by containing one of the following:

- 2.1 g/l MPG (equivalent to 0.08 C-mol per Liter)
- 2.1 g/l PEG 200 (equivalent to 0.08 C-mol per Liter) or

- 2.4 g/l Sucrose (equivalent to 0.08 C-mol per Liter)

Resulting in media with 2.1 g/l MPG; 2.1g/l PEG 200 or 2.4 g/l Sucrose, respectively.

By an error the media was prepared with 2.5 g/l MPG; 2.5 g/l PEG 200; and 2.5 g/l sucrose, respectively. This should therefore result in a lightly higher OD in these media compared to the intended media, if the compounds were easily metabolized.

The shake flasks were then incubated at 37°C at 300 rpm in 8 hr.

The OD (650 nm) was measured at 6 and 8 hr. and the following results were obtained:

Table 1. Test of MPG:

Average OD (650 nm) found for the three shake flask at time 6 and 8 hr.

OD (650 nm) Time [hr.]	OD_I	OD_{II}	OD_{III}	$\frac{(OD_{III}-OD_{II})}{(OD_I-OD_{II})}$
6	4.23	1.51	1.61	3.8%
8	4.08	1.46	1.57	4.3%

From the results it is clear that MPG is only very slowly or not at all metabolized by the strain used in this example. It is also clear that the culture is fully outgrown after 6 hr as the OD is not increased in medium I going from 6 to 8 hr.

Table 2. Test of PEG 200:

Average OD (650 nm) found for the three shake flask at time 6 and 8 hr.

OD (650 nm) Time [hr.]	OD_I	OD_{II}	OD_{III}	$\frac{(OD_{III}-OD_{II})}{(OD_I-OD_{II})}$
6	4.23	1.51	1.50	-0.2%
8	4.08	1.46	1.45	-0.4%

From the results it is clear that PEG 200 only very slowly or not at all metabolized by the strain used in this example.

Table 3. Test of sucrose:

Average OD (650 nm) found for the three shake flask at time 6 and 8 hr.

OD (650 nm) Time [hr.]	OD_I	OD_{II}	OD_{III}	$(OD_{III}-OD_{II})$ (OD_I-OD_{II})
6	4.23	1.51	4.23	100.1%
8	4.08	1.46	4.02	97.7%

From the results it is clear that sucrose is easy metabolized by the strain used in this example.

Example 2

Increased enzyme solubility in fermentation broth by addition of MPG to the fermentation process

Strain: *Bacillus licheniformis*

Polypeptide of interest: an alpha-amylase variant described in WO 01/66712

Media:

In all cases unless otherwise described tap water was used. All media were sterilized by methods known within the art to ensure that the fermentations were run as mono-cultures.

First inoculum medium:

LB agar: 10 g/l peptone from casein; 5 g/l yeast extract; 10 g/l Sodium Chloride; 12 g/l Bacto-agar adjusted to pH 6.8 to 7.2. Premix from Merck was used.

Transfer buffer:

M-9 buffer (deionized water is used): Di-Sodiumhydrogenphosphate, 2H2O 8.8 g/l; Potassiumdihydrogenphosphate 3 g/l; Sodium Chloride 4 g/l; Magnesium sulphate, 7H2O 0.2 g/l.

Inoculum shake flask medium (concentration is before inoculation):

PRK-50: 110 g/l soy grits; Di-Sodiumhydrogenphosphate, 2H2O 5 g/l; pH adjusted to 8.0 with NaOH/H3PO4 before sterilization.

Make-up medium (concentration is before inoculation):

Tryptone (Casein hydrolysate from Difco) 30 g/l; Magnesium sulphate, 7H2O 4 g/l; Di-Potassiumhydrogenphosphate 7 g/l; Di-Sodiumhydrogenphosphate, 2H2O 7 g/l; Di-Ammoniumsulphate 4 g/l; Citric acid 0.78 g/l; Vitamins (Thiamin-dichlorid 34.2 mg/l; Riboflavin 2.9 mg/l; Nicotinic acid 23 mg/l; Calcium D-pantothenate 28.5 mg/l; Pyridoxal-HCl 5.7 mg/l; D-biotin 1.1 mg/l; Folic acid 2.9 mg/l); Trace metals (MnSO4, H2O 39.2 mg/l;

FeSO4, 7H2O 157 mg/l; CuSO4, 5H2O 15.6 mg/l; ZnCl2 15.6 mg/l); Antifoam (SB2121) 1.25 ml/l; pH adjusted to 6.0 with NaOH/H3PO4 before sterilization.

Feed medium:

Glucose,1H2O 820 g/l;

Procedure for inoculum steps:

First the strain was grown on LB agar slants 1 day at 37°C.

The agar was then washed with M-9 buffer, and the optical density (OD) at 650 nm of the resulting cell suspension was measured.

The inoculum shake flask (PRK-50) is inoculated with an inoculum of OD (650 nm) x ml cell suspension = 0.1.

The shake flask was incubated at 37°C at 300 rpm for 20 hr.

The fermentation in the main fermentor (fermentation tank) was started by inoculating the main fermentor with the growing culture from the shake flask. The inoculated volume was 10% of the make-up medium (80 ml for 800 ml make-up media).

Fermentor Equipment:

Standard lab fermentors were used equipped with a temperature control system, pH control with ammonia water and phosphoric acid, dissolved oxygen electrode to measure >20% oxygen saturation through the entire fermentation.

Fermentation parameters:

Temperature: 41°C

The pH was kept between 6.8 and 7.2 using ammonia water and phosphoric acid

Control: 6.8 (ammonia water); 7.2 phosphoric acid

Aeration: 1.5 liter/min/kg broth weight

Agitation: 1500 rpm

Feed strategy:

0 hr. 0.05 g/min/kg initial broth after inoculation

8 hr. 0.156 g/min/kg initial broth after inoculation

End 0.156 g/min/kg initial broth after inoculation

Experimental setup:

Three fermentations were run in parallel all with the same inoculation material.

Fermentation A was run as described above.

Fermentation B was run as described above but 50 g/L MPG (monopropyleneglycol) was added to the make-up medium before inoculation.

Fermentation C was run as described above but 50 g/L MPG was added to the fermentation

24 hours after inoculation.

(In fermentation B and C, the concentrations of MPG is given as the concentration based on the volume of the make-up medium before inoculation.)

Samples were taken after 3 days of fermentation. The samples were split in two identical parts (sample I and sample II). Sample II was centrifuged at 15000 g_{av} in 20 minutes at 38°C. The resulting supernatant was then filtered through a 0.2µm filter (Sartorius Minisart, order no.: 16534) (sample II_{sup}).

The alpha-amylase activities in sample I and sample II_{sup} was then measured by methods known within the art (for example method for alpha-amylase activity measurements described in WO 95/26397 can be used). However, when measuring samples where the enzyme can be in a partly solid form, samples have to be treated with urea prior to analysis. By diluting the samples 1:50 (w/v) in a solution containing 40% (w/w) urea, 25.5 mg/L Brij 35 and 4.4 g/L CaCl2, 2H2O both crystalline and precipitated alpha-amylase is brought into solution in an active form and it can then be further diluted in the buffer used in the activity assay.

Both sample I and sample II_{sub} are diluted in the urea-buffer as described above before being analysed.

The enzyme activity in sample II_{sup} is a measure of the soluble activity, whereas the activity in sample I is a measure of the total activity (both soluble and crystallized and precipitated activity).

Results:

The following activities were found after 3 days of fermentation. The total activity found in fermentation A is used set to 100% and the soluble activity (i.e. the activity in sample II_{sup}) is given relative to the activity in sample I.

Table 4.

Fermentation name	Description	Enzyme activity in total broth Sample I	Enzyme activity in solution Sample II_{sup}
Fermentation A	Standard	100 %	19 %
Fermentaion B	Addition of MPG to make-up media	101 %	53 %

Fermentation C	Addition of MPG after 1 day of fermentation	99 %	95 %

From the results in Table 4 it is clear that addition of MPG to the media have a very significant effect on the amount of enzyme in solution in the fermentation broth.

EXAMPLE 3

Evaluation of the improved recovery Yield and Process

Two fermentations made in a scale larger than 50 liter were made with a *Bacillus* strain that produces an alpha-amylase with a low solubility. One of the fermentations had an addition of 2 % (w/w) MPG 24 hours after inoculation; here enzyme crystals were not visible in a microscope (40X). The other fermentation was a reference fermentation without any addition of MPG; here crystals were visible in the broth in a microscope (40X).

The two batches were treated equal in a series of flocculations where pH was adjusted to 4 different set points and all additions are in % w/w. The flocculation consisted of an addition of 200 % water; 2 % CaCl2; 1.2 % Al2(OH)5Cl; pH adjustment to a set point (see table below); 0.5 % Superfloc C591 and 0.2 to 0.3 % of Superfloc A130 depending of the pH. All flocculations were based on 100 g culture broth and all additions were done under stirring conditions. The flocculations were done at room temperature (approximately 20°C). The samples were centrifuged and the yield of the alpha-amylase activity were determined in the supernatant fractions.

Flocculation pH	**Supernatant yield MPG added to fermentation broth**	**Supernatant yield reference batch**
7.5	83 %	30 %
10.0	102 %	33 %
10.5	100 %	37 %
11.0	98 %	40 %

Table 5: Flocculation at different pH to illustrate the effect of MPG added to the fermentation on supernatant (centrate) yield.

In an industrial scale the sludge from the first centrifugation would be reflocculated and centrifuged again to increase the total separation yield. The sludge from the flocculation trials with pH 7.5 (mentioned above) was resuspended in 150 % water and 1 % CaCl2 and finally flocculated with 0.1 to 0.2 % Superfloc A130. Initially the sludge from the flocculation based on the broth without MPG added to the fermentation had 3x more activity than the sludge that came from flocculation based on the fermentation with MPG added to it. Again crystals were visible in a microscope (40X) in the sludge coming from the fermentation without MPG addition whereas the sludge from the batch coming from the fermentation with MPG did not contain any visible crystals in a microscope (40X).

Again the samples were centrifuged and the yield of the alpha-amylase activity in the reflocculated sludge was determined in the supernatant fractions.

Flocculation pH	Supernatant yield MPG added fermentation broth	Supernatant yield reference batch
7.5	52 %	6 %
10.0	61 %	7 %
10.5	64 %	10 %
11.0	67 %	10 %

Table 6: Flocculation of sludge from first flocculation at different pH to illustrate the effect of MPG added to the fermentation.

As illustrated in this example the yield is much higher in the recovery of the enzyme if the crystal formation can be reduced or eliminated by addition of MPG during fermentation.

EXAMPLE 4

Effect of adding both MPG and a mineral salt to the fermentation broth

Materials and methods:

A fermentation method similar to the one described in Example 2 (fermentation A) was used as the basis for these experiments.

Table 7. Experimental setup:

Fermentation name	Addition of MPG after 1 day of fermentation [1)]	Extra mineral added [2)]	Amount [3)]	
			[g/l]	[mol/l]
D	Yes	No	-	-
E	Yes	NaCl	12	0.21
F	Yes	KCl	15	0.20
G	Yes	Na_2SO_4	14	0.10
H	Yes	K_2SO_4	18	0.10
I	No	NaCl	12	0.21

1) This indicates if MPG is added to the fermentation 1 day after inoculation. The amount of MPG added is 50 g/l MPG based on the volume of the make-up media before inoculation.

2) The extra mineral or extra amount of an already added mineral added to main media before inoculation.

3) Salt concentration, amounts are based on volume of the make-up media before inoculation.

Experimental setup:

Fermentation D is the reference process for this study, while the fermentations E, F, G, H, I had extra mineral salt added to the make-up media in the amounts found in table 7. Samples were taken after 3 days of fermentation. The samples were split into two identical parts (sample I and sample II). Sample II was centrifuged at 15000 g_{av} in 20 minutes at 38°C. The resulting supernatant was then filtered through a 0.2µm filter (Sartorius Minisart, order no.: 16534) (sample II_{sup}).

The alpha-amylase activities in sample I and sample II_{sup} was then measured by methods known within the art (for example method for alpha-amylase activity measurements described in WO 95/26397 can be used). However, when measuring samples where the enzyme can be in a partly solid form, samples have to be treated with urea prior to analysis. By diluting the samples 1:50 (w/v) in a solution containing 40% (w/w) urea, 25.5 mg/L Brij 35 and 4.4 g/L CaCl2, 2H2O both crystalline and precipitated alpha-amylase is brought into solution in an active form and it can then be further diluted in the buffer used in the activity assay.

Both sample I and sample II_{sub} were diluted in the urea-buffer as described above before

being analysed.

The enzyme activity in sample II_{sup} is a measure of the soluble activity, whereas the activity in sample I is a measure of the total activity (both soluble and crystallized and precipitated activity).

Results:

Table 8: The following activities were found after 3 days of fermentation. The total activity found in fermentation D is set to 100%, and the soluble activity (i.e. the activity in sample II_{sup}) is given relative to the activity in sample I for the specific fermentation.

Fermentation name	Description	Enzyme activity in total broth Sample I	Enzyme activity in solution Sample II_{sup}
Fermentation D	Addition of MPG after 1 day of fermentation (reference)	100	39
Fermentaion E	Type D + NaCl in makeup	96	156
Fermentation F	Type D + KCl in makeup Type	88	149
Fermentation G	Type D + Na2SO4 in makeup	99	148
Fermentation H	Type D + K2SO4 in makeup	82	152
Fermentation I	NaCl in makeup, no MPG	109	15

The reason for the activity being higher in sample II, compared to sample I in some cases is that in the cases where all enzyme is in solution, removal of the cells increases the enzyme concentration in the sample, as the cells have a significant volume, but only a low enzyme concentration.

The data in table 8 clearly show that the increased enzyme solubility achieved by the MPG addition (D versus I : 39% versus 15%; and E versus I: 156 % versus 15 %). It is also clear that the increased solubility of the enzyme achieved by the MPG addition can be enhanced

by the addition of various minerals.

Table 8 shows that there is a synergistic effect (see Fermentation D (MPG added); Fermentation I (NaCl added); and Fementation E (MPG and NaCl added)).

EXAMPLE 5

A solution of alpha-amylase (e.g. recovered from the broth described in Example 2 by conventional means) is concentrated at pH 10.5-11 at 40°C. The concentrate is filtered through a 0.2µm filter at 40°C. Aliquots of 25 mL is transferred to 7 vials, to which is then added one of the following polyols:

Vial no.	Polyol
1	reference
2	5% (w/w) PEG 200
3	5% (w/w) PEG 400
4	5% (w/w) sorbitol
5	5% (w/w) cellobiose
6	5% (w/w) xylitol
7	5% (w/w) monopropylene glycol (MPG)

A magnetic bar is placed in each vial, and the pH is adjusted to pH 7.5. The vials are then left at room temperature with slow stirring for two days.

The crystals are then removed by filtration (0.2 µm filters) and the activity is measured both in the filtrate (= mother liquor) and in the sample before filtration. The results presented in the table below are given as the activity in the mother liquor relative to the total activity in the sample before filtration (in %).

Table 9.

Vial no.	Polyol added	Activity in mother liquor relative to total activity (%)
1	Reference	9
2	5% (w/w) PEG 200	58
3	5% (w/w) PEG 400	53
4	5% (w/w) sorbitol	33
5	5% (w/w) cellobiose	38
6	5% (w/w) xylitol	29
7	5% (w/w) monopropylene glycol (MPG)	29

The data clearly demonstrate that addition of polyols increases the solubility of the amylase.

CLAIMS

1. A method for fermenting a microorganism, producing a polypeptide of interest, in a culture medium of at least 50 litres, comprising:
adding one or more compounds selected from the group consisting of 1,2-propandiol, 1,3-propandiol, ethylene glycol, trehalose, xylitol, arabitol, dulcitol, mannitol, erythritol, cellobiose, sorbitol and a polyether having an average molecular weight less than 1000, to the culture medium before and/or during fermentation, wherein the compound is low metabolizable measured by $(OD_{III}-OD_{II})/(OD_{I}-OD_{II}) < 25\%$ as defined herein.

2. The method according to claim 1, wherein the microorganism is a bacterium or a fungus.

3. The method according to claim 2, wherein the bacterium is a *Bacillus* strain.

4. The method according to claim 1, wherein the polypeptide is a protein or a peptide.

5. The method according to claim 1, wherein the polypeptide is an enzyme, in particular a hydrolase (class EC 3 according to Enzyme Nomenclature).

6. The method according to claim 4, wherein the peptide contains from 2 to 100 amino acids.

7. The method according to claim 1, wherein the compound is added in an amount of least 0.1 % (w/w) of the culture medium.

8. The method according to claim 1, wherein the compound is 1,2-propandiol.

9. The method according to claim 1, wherein in addition to the compound a salt is added to the fermentation medium.

10. The method according to claim 9, wherein the salt is selected from the group consisting of a chloride, a sulphate, a phosphate, a nitrate, and an ammonium salt.

11. The method according to claim 1, wherein the polypeptide of interest is recovered.

12. The method according to claim 1, wherein the polypeptide is recovered after removal of

the microorganism.

13. A method for fermenting a microorganism, producing a polypeptide of interest, in a culture medium of at least 50 litres, comprising:

adding one or more compounds selected from the group consisting of 1,2-propandiol, 1,3-propandiol, ethylene glycol, trehalose, xylitol, arabitol, dulcitol, erythritol, cellobiose, and a polyether having an average molecular weight less than 1000.