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— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
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(54) Title: SOPHOROLIPIDS AS PROTEIN INDUCERS AND INHIBITORS IN FERMENTATION MEDIUM

(57) Abstract: A method for producing sophorolipids having protein inducer and/or repressor activities having the steps of synthesizing the sophorolipid by fermentation of *Candida bombicola* in a fermentation media to form a natural mixture of lactonic sophorolipids and non-lactonic sophorolipids and then utilizing the natural mixture as a protein inducing agent, utilizing the natural mixture as a protein repressing agent, and/or utilizing the natural mixture as a combined protein induction/repressor agent. An application of the sophorolipid compound produced according to the method as a microbial media component.

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SOPHOROLIPIDS AS PROTEIN INDUCERS AND INHIBITORS IN FERMENTATION MEDIUM

STATEMENT OF RELATED APPLICATIONS

This patent application is based on and claims priority on US Provisional Patent Application No. 60638747 field on 22 December 2004.

BACKGROUND OF THE INVENTION

1. Technical Field.

The present invention in general relates to the field of uses for sophorolipids and more specifically to the field of protein inducers and as a media component. The invention is directed to developing low cost inducers/repressors for protein production.

2. Prior Art.

For production of proteins on a larger scale, a need exists for cheap and well defined molecules to act as inducers of the protein of interest and also as a repressor for unwanted enzymes. This aids in higher yields, decreasing the purification costs and enhancing the profits. Sophorose has been shown to be a good inducer of cellulase protein. See Hrmova, M., Petrakova, E., Biely, P., Journal of General Microbiology 137, 541-547 (1991). However, the cost of the molecule makes it impractical to be used at commercial level.

Sophorolipids are microbial extracellular glycolipids produced by resting cells of *Candida bombicola*. The chemical composition of sophorolipid is constituted by a disaccharide sugar viz. sophorose and a fatty acid or an ester group. *Candida bombicola* produces the sophorolipids as a mixture of macroloctones and free acid structures that are acetylated to various extents at the primary hydroxyl sophorose ring positions (FIG. 1). See Bisht, K.S. et al., J. Org. Chem., vol. 64, pp. 780-789 (1999).

It is to the development of sophorolipids for production and industrial purposes, and other purposes, that the present invention is directed.

BRIEF SUMMARY OF THE INVENTION

A natural mixture of sophorolipids was synthesized by fermentation of *Candida bombicola*. When *B. subtilis* was grown in presence of sophorolipids, increased production of amylase was observed. When *Pleurotus ostreatus* 473 strain, which produces both laccase and manganese peroxidase, was grown in the presence of sophorolipids, increased production of laccase was observed while the production of manganese peroxidase decreased.

Thus, the applications of sophorolipids in the fields of industry and basic sciences would have tremendous advantages in higher production or inhibition rates of proteins. Further, while sophorolipids can find applications in diversified fields, the present invention indicates that sophorolipids could be used as protein inducers and repressors.

These features, and other features and advantages of the present invention, will become more apparent to those of ordinary skill in the relevant art when the following detailed description of the preferred embodiments is read in conjunction with the appended drawing.

BRIEF DESCRIPTION OF THE FIGURE

FIG. 1 are representative structures of sophorolipids produced by *Candida bombicola*, with FIG. 1a showing lactonic sophorolipid and FIG. 1b showing open ring sophorolipid.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

1. Sophorolipid Fermentation

Sophorolipids were synthesized by fermentation of *Candida bombicola*. The fermentation media was composed of glucose 100 g, yeast extract 10 g, urea

1 g and oleic acid 40 g in 1000 ml of water. After 7 days of fermentation, sophorolipid was extracted thrice using ethyl acetate. The extracts were pooled and the solvent then was removed. The obtained product was therein washed with hexane to remove the residual fatty acids. This was "natural" sophorolipid. The sophorolipid was dried in a vacuum desiccator.

2. Study of amylase protein induction

Bacillus subtilis strain was used to study the effect of sophorolipid on amylase induction. Culture was grown on Peptone: 5gm/L, Beef extract: 3gm/L and Starch: 2gm/L. A single colony of *Bacillus subtilis* was inoculated in 10ml medium and was allowed to grow for 12 hours. After the culture reached to the log phase, 1ml of the same was inoculated in 100ml of the medium. Control experiments having no sophorolipids also were conducted, whereas the experimental set has 1mM of sophorolipid prepared in DMSO.

After 48 hours a sample was withdrawn, centrifuged at 8000 rpm for 20 minutes, and amylase activity determined using standard methods.

3. Study of laccase protein induction and Manganese peroxide repression

Pleurotus ostreatus 473 was used to study the effect of sophorolipids on Laccase/Manganese peroxidase production. Culture was cultivated in agar medium containing 5 g/L cellulose, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L ammonium tartrate, 0.2 g/L NaH_2PO_4, 50 mg/L $CaCl_2$, 50 mg/L, $FeSO_4 \cdot 7H_2O$, 10 mg/L $CuSO_4 \cdot 5H_2O$, 5 mg/L $ZnSO_4 \cdot 7H_2O$, 5 mg/L $MnSO_4 \cdot 4H_2O$ and 25 g/L agar. pH of the medium was 6.0. For enzyme production studies, flasks with 20 mL of the above media (without agar) were inoculated with two wort agar plugs (2° Balling, 10 mm diameter), cut from the actively growing part of a colony on a Petri dish, and incubated at 27°C for 14 days. After 14 days a sample was withdrawn, centrifuged at 8000 rpm for 20 minutes and laccase and MnP activity determined using standard methods.

Results and Discussion

As shown in Table 1, upon growing *B. subtilis* in the presence of sophorolipid, an increase of 39% amylase protein production observed. Growing *Pleurotus ostreatus* 473 in the presence of 1mM sophorolipid natural mixture resulted in 4.5 times increase in laccase protein whereas there was a decrease of 25% in the production of manganese peroxide protein. This shows that the sophorolipid natural mixture has the ability to act as an inducer for some proteins and the ability to act as a repressor for others.

The invention will find its place in industries that produce protein products such as enzymes through microbial fermentation. Also, microbial media that has sophorolipids as a component can be sold for protein induction studies.

Sophorolipids will be administered in the fermentation medium as either fine powder or in the form of solutions prepared in DMSO, alkaline sucrose solution, chloroform, methanol or ethyl acetate.

Table 1: % Increase/decrease of enzyme production

Enzyme	% values
Laccase	
Control	**100**
Sophorolipid Induced	**453**
Manganese peroxidase	
Control	**100**
Sophorolipid Induced	**73**
Amylase	
Control	**100**
Sophorolipid induced	**139**

The foregoing detailed descriptions of the preferred embodiments and the appended figure have been presented only for illustrative and descriptive purposes and are not intended to be exhaustive or to limit the scope and spirit of the invention. The embodiments were selected and described to best explain the

principles of the invention and its practical applications. One skilled in the art will recognize that many variations can be made to the invention disclosed in this specification without departing from the scope and spirit of the invention

CLAIMS

What is claimed is:

1. A method for producing sophorolipids having protein inducer and/or repressor activities comprising the steps of:
   a. synthesizing the sophorolipid by fermentation of *Candida bombicola* in a fermentation media to form a natural mixture of lactonic sophorolipids and non-lactonic sophorolipids; and
   b. utilizing the natural mixture as a protein inducing agent.

2. A method for producing sophorolipids having protein inducer and/or repressor activities comprising the steps of:
   a. synthesizing the sophorolipid by fermentation of *Candida bombicola* in a fermentation media to form a natural mixture of lactonic sophorolipids and non-lactonic sophorolipids; and
   b. utilizing the natural mixture as a protein repressing agent.

3. A method for producing sophorolipids having protein inducer and/or repressor activities comprising the steps of:
   a. synthesizing the sophorolipid by fermentation of *Candida bombicola* in a fermentation media to form a natural mixture of lactonic sophorolipids and non-lactonic sophorolipids; and
   b. utilizing the natural mixture as a combined protein induction/repressor agent.

4. An application of the sophorolipid compound produced according to Claim 1 as a microbial media component.

5. An application of the sophorolipid compound produced according to Claim 2 as a microbial media component.

6. An application of the sophorolipid compound produced according to Claim 3 as a microbial media component.

7. An application of the sophorolipid compound produced according to Claim 1 as a protein inducer added to microbial media during protein production.

8. An application of the sophorolipid compound produced according to Claim 2 as a protein repressor added to microbial media during protein production.

9. An application of the sophorolipid compound produced according to Claim 3 as a protein inducer and repressor added to microbial media during protein production.

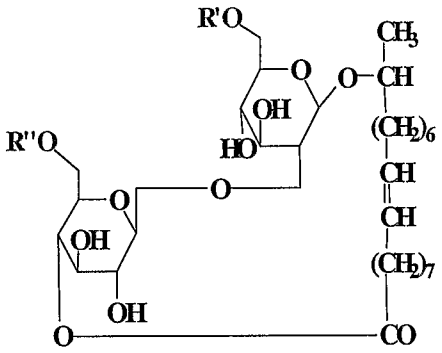
10. The application of the sophorolipid compound produced according to Claims 4 through 9 wherein the protein is an enzyme.

11. The application of the sophorolipid compound produced according to Claims 4 through 9, wherein the enzyme is amylase.

12. The application of the sophorolipid compound produced according to Claims 4 through 9, wherein the enzyme is laccase.

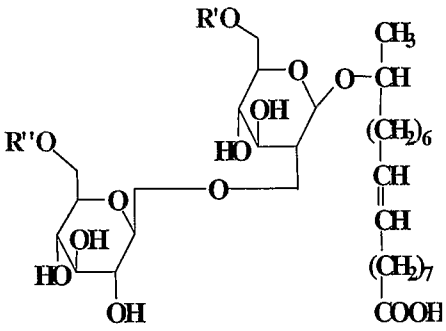
13. The application of the sophorolipid compound produced according to Claims 4 through 9, wherein the enzyme is manganese peroxidase.

14. The application of the sophorolipid compound produced according to Claims 4 through 9, wherein there are two enzymes and the enzymes are laccase and manganese peroxidase.



1. R' = R'' = Ac or H
2. R' = Ac; R'' = H
3. R' = H; R'' = Ac
4. R' = R'' = H

a. Lactonic sophorolipid



5. R' = R'' = Ac or H
6. R' = Ac; R'' = H
7. R' = H; R'' = Ac
8. R' = R'' = H

b. Open ring sophorolipid

FIG. 1: Structures of sophorolipids produced by *Candida bombicola.*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/46457

A. CLASSIFICATION OF SUBJECT MATTER
IPC: **C12P 19/00**(2006.01)

USPC: 435/72
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,616,479 (MARCHAL et al) 01 April 1997 (01.04.1997), abstract, Background and summary of the invention, examples, and claims.	1-14
Y	US 5,756,471 (HILLION et al) 26 May 1998 (26.05.1998), abstract, columns 1-3, examples, and claims.	1-14
Y	US 5,767,255 (WULLBRANDT et al) 16 Jun 1998 (16.06.1998), abstract, summary of the invention, column 3, 6, examples, and claims.	1-14
Y	US 6,433,152 B1 (LANG et al) 13 Aug 2002 (13.08.2002), abstract, Background and Summary of the invention, columns 4-5, and claims.	1-14
Y	US 2004/0171512 (FURUTA et al) 02 Sept 2004 (02.09.2004), abstract, Disclosure of the invention, page 2, Industrial applicability.	1-14
Y	DAVILA A.-M. et al. Sophorose lipid fermentation with differentiated substrate supply for growth and production phases, Applied Microbiology and Biotechnology, 1997, vol 47, pages 496-501, especially abstract, introduction, and materials & methods.	1-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

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International application No.
PCT/US05/46457

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BRAKEMEIER A. et al. Candida bombicola: production of novel alkyl glycosides based on glucose/2-dodecanol, Applied Microbiology and Biotechnology, 1998, vol 50, pages 161-166, especially abstract and materials & methods.	1-14
Y	RAU U. et al. Sophorolipids: a source for novel compounds, Industrial Crops and Products, 2001, vol 13, pages 85-92, especially abstract, materials & methods, and conclusions.	1-14
Y	GROSS R.A. et al. Glycolipids from Torulopsis bombicola: Biosynthesis, lipase-selective modification and anti-cancer activity, Abstract, 217th American Chemical Society National Meeting, Anaheim CA, March 21-25, 1999, BIOT-181, ACS; Washington, DC.	1-14
Y	HRMOVA et al. Induction of cellulose- and xylan-degrading enzyme systems in Aspergillus terrus by homo- and heterodisaccharides composed of glucose and xylose, Journal of General Microbiology, 1991, vol 137, no 3, pages 541-547, especially abstract.	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/46457

Continuation of B. FIELDS SEARCHED Item 3:
EAST:
USPAT, USOCR, PG-PUB, JPO, EPO, DERWENT,
STN: CAPLUS, BIOSIS, MEDLINE, AGRICOLA, CONFSCI, EMBASE, PCTFULL, WPIDS

SEARCH STRATEGIES:
sophoro? or sophorolipid? or sophoroside
candida bombicola and ferment? or cultur?
induc? or regulat? or control? or inhibit? or stimulat? and protein?
amylase or laccase or peroxidase and ferment? and sophorolipid?