



COMPARATIVE STUDY FOR THE PRODUCTION, CHARACTERISATION AND ANTIMICROBIAL STUDIES OF SOPHOROLIPIDS USING *CANDIDA TROPICALIS*

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ABSTRACT

Sophorolipids are biosurfactants which consists of a dimeric carbohydrate sophorose linked to long-chain hydroxy carboxylic acids. After optimization of growth and cultural conditions an ideal condition for Sophorolipids production in a small scale bench top fermenter (Murhopye scientific, LF) was designed. The ideal production medium for the production of sophorolipids for fermentation was containing glucose, yeast extract, urea and oleic acid. The ideal cultural conditions like temp, ph, incubation period and RPM. For comparative study the fermentation was carried out using batch culture, fed batch-I and fed batch-II for obtaining maximum yield. Extraction was carried out using different solvents for product recovery. The maximum yield of sophorolipids (SLs) was obtained in fed-batch-II. Characterization of sophorolipids was carried out by determining surface tension and CMC. The antimicrobial studies showed positive results.

Keywords: Production medium, extraction, characterization, antimicrobial studies.

INTRODUCTION

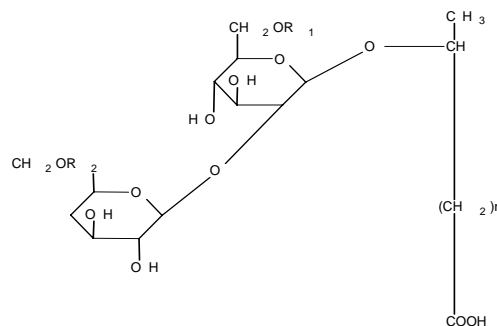
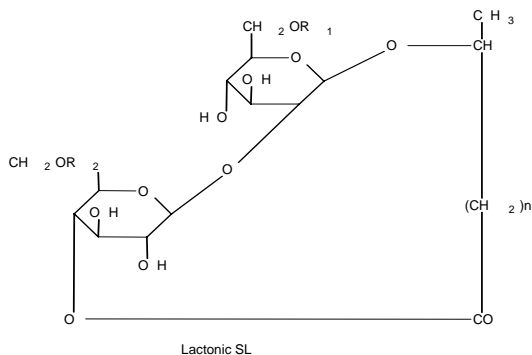
Sophorolipids

Sophorolipids are biosurfactants which consists of a dimeric carbohydrate sophorose linked to long-chain hydroxy carboxylic acids. They are usually a mixture of at least six to nine different hydrophobic sophorolipids. Yeasts have been shown to be potent producers of sophorolipids type of biosurfactants. The sugar unit is the disaccharide sophorose which consists of two β -1, 2-linked glucose units. The 6 and 6' hydroxy groups are generally acetylated ¹. *Torulopsis bombicola* produces a Sophorolipids like biosurfactant during alkane fermentation ² while *Torulopsis petrophilum* and *Torulopsis apicola* are major producers of sophorolipids ^{3,4}.

A surface active glycolipid from *Candida petrophilum* grown on hydrocarbons while *Candida bogoriensis* produces a glycolipid in which sophorose is linked to decosanoic acid diacetate ⁵.

Organism Profile

Candidas are ascomycetous or basidiomycetous fungi that reproduces vegetatively by budding or fission and that forms sexual status which are not enclosed in a fruiting body. The subdivisions are based on aspects of *Candida* sexuality (Ascomycotina or Basidiomycotina) or lack of it (Deutromycotina). Various *Candida spp* come under the subdivision deutromycotina, *Candida* are anamorphic i.e., Mitosporic expression.



(R1 and R2 = Ac or H; n = 13 or 15; fatty acid chain may include double bonds)

MATERIALS AND METHODS

Candida tropicalis

Macroscopic Morphology

On sabouraud dextrose agar, *Candida tropicalis* colonies are cream coloured with a slightly mycelial border. They are smooth, glabrous and yeast like in appearance ^{6,7}. It may produce a thin surface film and bubbles when grown in sabouraud broth ⁸.

Microscopic Morphology

Microscopic morphology shows spherical to subspherical budding yeast like cells or blastoconidia, 3.0-5.5 X 4.09 - 9.0 μ m in size. On Cornmeal tween 80 agar plate they are abundant long, wavy, branched Pseudohyphae with numerous ovoid blastoconidia budding off. Terminal vesicles (Chlamydoconidia) are not produced.

On cornmeal tween 80 agar and at 25°C after 72 hours, it produces oval blastospores which are located along the long pseudohyphae. The blastospores may appear singly or in clusters. The pseudohyphae branch abundantly. *Candida tropicalis* may also produce true hyphae.

India ink preparation: Negative - No capsules present.

Physiological tests: Germ tube test shows negative, Hydrolysis of Urea shows positive, Growth on Cyclohexidine medium shows positive, Growth at 37°C shows positive.

Fermentation Reactions

Where fermentation means the production of gas and is independent of pH changes. Positive: Glucose; Maltose; Galactose; Trehalose (delayed), Variable: Sucrose, Negative: Lactose.

Assimilation tests:

Positive: Glucose; Galactose; Maltose; Trehalose; D-Xylose, Soluble starch, Succinic acid; D-Mannitol; L-Arabinose(weak); D-Glucitol

Variable: Sucrose; Salicin; Melezitose; Glycerol; Cellobiose; D-Ribose; Ribitol; L-Sorbose; Citric acid; DL-Lactic acid

Negative: Potassium nitrate; L-Rhamnose; Lactose; Raffinose; Melibiose; Galactitol; Erythritol; Inositol; D-Arabinose

Clinical Significance

Candida tropicalis is a major cause of septicemia and disseminated candidiasis, especially in patients with lymphoma, leukaemia and diabetes. It is the second most frequently encountered medical pathogen, next to *C. albicans* and is also found as part of the normal human mucocutaneous flora. Sucrose negative variants of *C. tropicalis* have also been increasingly found in cases of disseminated candidiasis. Environmental isolations have been made from faeces, shrimp, kefir and soil.

Mycosis: Candidiasis [9].

General methods used in production of biosurfactants:

Biosurfactant Production:

The optimized production medium, cultural condition for the production of sophorolipids was carried by Sudha.S, et.al, [10, 11]. The production of biosurfactants by micro organisms can be during exponential growth or it may be during the stationary phase of growth when the nutrient limiting conditions start prevailing in the growth medium. In case of growth associated biosurfactant production, there exists a parallel relationship between substrate utilization, growth and biosurfactant production.

The production of AP-6, a glycoprotein type of biosurfactant by *Pseudomonas fluorescens* 378 is growth associated. Similarly, the production of emulsan in *A. caloaceticus* and the fermentative production of surface active agent from *Bacillus aerus* IAF-346 and *Bacillus species* IAF 343 were found to be growth associated. The production of rhamnolipids by *Pseudomonas aeruginosa* is also related to growth. There are several methods in the production of biosurfactants. They are Batch method and Fed batch method.

Batch Method

In this method after adding the inoculum to the medium, the fermentation was carried out continuously till the end of fermentation.

Fed-Batch Method

In this method after adding the inoculums, the fermentation was carried out and the feed was added every 24 hrs till the end of fermentation.

Types of General Extraction Procedures

After the biosurfactant has been produced comes the question of its recovery. The choice of method for recovery of a particular biosurfactant depends on its ionic charge, solubility in water, whether the product is cell bound or extracellular and of course, the cost of recovery. The methods generally used for biosurfactant recovery include solvent extraction, adsorption followed by solvent extraction, precipitation, crystallization, centrifugation and foam fractionation. Most biosurfactants are secreted into the medium and thus are isolated from either culture filtrate or supernatant obtained after removal of cells. Various methods employed for recovery of biosurfactants can be broadly classified under two categories. The

ones involved in batch recovery and the ones involved in continuous recovery [12].

Common methods employed for recovery of biosurfactants:

Batch Recovery

Solvent extraction (Chloroform-methanol), Acetone, Ethyl acetate, Hexane, Crystallization, Precipitation (a. Ammonium sulphate, b. Acid, c. Acetone)

Continuous Recovery

Centrifugation, Foam separation and precipitation, Diferlation and precipitation, Adsorption, Tangential flow filtration

Sophorolipids Fermentation

The ideal condition for sophorolipids production in a small scale bench top fermenter (Murhopye scientific, LF) with medium and cultural conditions are showed in Table 1 & 2.

Table 1: Production Medium

Parameters	Optimized
Temp	30°C
pH	3.5
Incubation period	7 days
Rpm	100-120

Table 2: Cultural Conditions

Ingredients	Quantity (g/lit)
Glucose	100
Yeast extract	10
Urea	1
Oleic acid	40

Table 3: Fermentation of SLS by *Candida tropicalis*

Process Strategy	Yield in g/g of glucose	Initial glucose/lit	Yield of SLS g/lit
Batch culture	0.385	100	38.5
Fed Batch - I	0.402	100	40.2
Fed Batch - II	0.498	100	49.8

Organism Used: *Candida tropicalis*

Fermentation of Sophorolipids

Batch Culture

Before inoculation the production medium was supplemented with 100 g of glucose, 10 g of yeast extract and 1 g of urea. The medium was sterilized in an autoclave before inoculation, 35.56ml of oleic acid (filter sterilized) was added to the medium after sterilization 100 ml of inoculum was added to the 1000 ml of production medium and the fermentation was carried out at 30°C, 100-120 rpm at a constant pH 3.5 for 7 days. The fermentation was monitored by aseptically removing 30 ml of the sample every 24 hours of the duration of experiment. Analysis of the sample for surface tension and CMC measurement was done on the samples. After 7 days the fermentation medium was removed and extraction was carried out.

Fed Batch Culture - I

The above-mentioned procedure as in batch culture was performed and after 3 days, 40g of glucose was added every 24 hrs till the 7th day. Then analysis of the sample for surface tension and CMC measurement was done as in the above procedure and the extraction was carried out.

Fed Batch Culture - II

The above-mentioned procedure as in batch culture was performed and after 3 days 40 g of glucose and 20 g of oleic acid was added every 24 hrs till the 7th day. Analysis of the sample for surface tension and CMC measurement was done as in the above procedure and the extraction was carried out.

Extraction of Sophorolipids

To isolate sophorolipids the entire culture (cells and broth) was lyophilized for 8 hours. The dried residues were divided into six portions. Each portion was extracted with ethyl acetate (500 ml) by shaking at 30°C for 5 days. The extraction mixture was filtered through Whatman No. 2 filter paper, and the residues were rinsed twice with ethyl acetate (500 ml each time). The combined filtrate was concentrated by evaporation and added to 1,1 hexane/petroleum ether (90/10 v/v) to precipitate out the pure sophorolipids. After vacuum drying in desiccators, the sophorolipids were weighed ¹³.

Characterisation of Sophorolipids

The characterizations of sophorolipids were done by determining the surface tension, and critical micelle concentration (CMC) ¹⁴.

Determination of Surface Tension

30 ml of the medium containing the microbial surfactant was used for determining the surface tension. The surface tension was calculated using the formula.

$$r_2 = \frac{n_2 P_1}{n_1 P_2} x r_1$$

Where,

r₁= Surface tension of water at 30°C, 7.18 dynes/cm

r₂= Surface tension of test liquid (medium containing the microbial surfactant)

n₁= No. of drops of test liquid (medium containing the microbial surfactant)

n₂= No. of drops of water

p₁= Density of test liquid (medium containing the microbial surfactant)

P₂= Density of water at 30°C, 0.9956 g/cc

The density of test liquid was determined by the formula

$$P_1 = \frac{W_3 - W_1}{W_2 - W_1} x P_2$$

Where

W₁= Weight of pyknometer in grams

W₂= Weight of pyknometer + water in grams

W₃= Weight of pyknometer + test liquid in grams

P₁= Density of test liquid

P₂= Density of water at 30°C, 0.9956 g/cc

Determination of Critical Micelle Concentration (CMC)

Initially 2% stock solution was prepared by dissolving 2 ml of the partially purified microbial surfactant in 100ml of distilled water. Then 0%, 0.02%, 0.04%, 0.06%, 0.08%, 0.1%, 0.2%, and 0.3% were obtained. By taking 0, 1, 2, 3, 4, 5, 10 and 15 ml of stock solution and making it upto 100 ml in standard flask. The surface tension of each solution was determined using Stalagmometer as illustrated in previous section. The results were shown in table. The concentration of the microbial surfactant at which it begins to form micelles was known as critical micelle concentration ¹⁵.

Microbial Studies

Anti-microbial activity of crude sophorolipids obtained from *Candida tropicalis* was determined using disc diffusion procedure

Organisms Used: *E.coli*, *Bacillus subtilis*

Disc diffusion method allows for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc.

Procedure: Nutrient agar medium was prepared and poured into petridish and allowed to solidify. After solidification, culture of test

organism was aseptically spread over the solidified agar medium using swabbing technique. The antibiotic discs were placed over the divided quadrants and they were gently tapped to fix it to the medium. The plates were incubated at 37°C for 24 hrs to 48hrs and observed for the zone of inhibition ¹⁶.

Antifungal activity of crude sophorolipids obtained from *Candida tropicalis* was determined using disc diffusion procedure

Organisms Used: *Candida albicans*, *Aspergillus niger*

Disc diffusion method allows for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc.

Procedure

Sabouraud dextrose agar medium was prepared and poured into petridish and allowed to solidify for bacteria and sabouraud dextrose agar for fungi. After solidification, culture of test organism was aseptically spread over the Solidified sabouraud dextrose agar medium using swabbing technique. The antifungal discs were placed over the divided quadrants and they were gently tapped to fix it to the medium. The plates were incubated at 37°C for 24 hrs to 48hrs and observed for the zone of inhibition ¹⁶.

RESULTS AND DISCUSSION

Sophorolipids Production:

The ideal condition for sophorolipids production in a small scale bench top fermenter (Murhopye scientific, LF) with medium containing glucose-100 g/L, yeast extract-10g/L, urea-10g/L and oleic acid-40 ml/L. In our study Fed-batch-II fermentation gave maximum of 49.8 g/L of Sophorolipids from *Candida tropicalis*. Whereas batch culture and Fed- batch-I produced 38.5 g/L and 40.2 g/L respectively.

Characterization of sophorolipids

Characterization of sophorolipids was done by determining surface tension and CMC. The minimum surface tension of the crude sophorolipids from *Candida tropicalis* in buffered solution (pH 6) was measured as 34 m Nm⁻¹ and the CMC value of obtained sophorolipids was 19.2 mg/L at pH 6.

Microbial Assays:

Antimicrobial activity

Antibacterial activity and antifungal activity of *Candida tropicalis* were done using disc diffusion procedure. There was zone of inhibition after 24 hours of incubation indicating the separated sophorolipids are having antibacterial and/or antifungal activity.

DISCUSSIONS

Many authors studied on sophorolipids (Glycolipid type of biosurfactants) only from *Candida bombicola* ATCC 22214. This is the first study on production of sophorolipids from *Candida tropicalis* by using medium containing 100 g/L glucose, 10 g/L yeast extract, 10 g/L urea and 40 ml/L oleic acid. From the cultural studies we found that an optimum pH for sophorolipids production from *Candida tropicalis* was found to be at pH 3.5, any change to both lower and higher cost an appreciable drop in Sophorolipids production. An optimum temperature was found to be 30° C. Glucose was almost completely consumed at all temperatures tested. The property of surfactants was not affected by different incubation temperatures. An ideal incubation period was found to be 7 days for maximum yield and also 200 rpm, speed of agitation was found to be an optimum for *Candida tropicalis* from the study of Sophorolipids production, we obtained higher yield when carbon and lipid sources were supplied constantly throughout the incubation period.

CONCLUSION

In recent years microorganisms have found their applications not only in the production of a variety of metabolites but also in the bio

transformation of several chemicals. Now a day's Sophorolipids (Glycolipid biosurfactants) is used as a source for preparing new compounds of useful functionality such as glucose lipid -acid [17-L-(β -glucopyranosyl)-oxy- octadec-(9)-enoic acid]which are commercially not yet available and difficult to prepare employing organic synthesis, could be a useful intermediate for polyester and macrocyclic lactone production. These sophorolipids can be used for recovery of metals for polluted water ways and use of bio fertilizers and biopesticides. From our study we obtained 49.8g/L Sophorolipids from Fed-batch -II method using *Candida tropicalis*. The yield can be improved by mutations and also the cost of sophorolipids can be reduced by using cheap substrates such as sugar molasses, meat molasses and waste meat product, sulphur waste.

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