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PRODUCTION, PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF BIOSURFACTANT PRODUCED BY *CANDIDA TROPICALIS* VEMS 27

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ABSTRACT

Nowadays, biosurfactants are one among the most important biochemical compound required for many industrial applications. So, the need for medium optimization is significant for enhanced production, this study used a potential biosurfactant producing estuarine yeast, Candida tropicalis VEMS 27 to accomplish the stated aim. This study optimized various physicochemical factors under shake flasks conditions and showed enhanced biosurfactant production at 42 h incubation with 2% of glucose, 1.5% of ammonium nitrate as carbon and nitrogen sources with pH 7, 35°C temperature and 10pp salinity. Under standardized conditions, the large-scale production was carried out and the biosurfactant was extracted with acid precipitation procedure and purified using silica gel column chromatography. The final dry weight of the purified biosurfactant was accounted as 4.85g/L and was biochemically characterized as glycolipid using TLC method. Thus, from the present study, the estuarine yeast C. tropicalis VEMS 27 proved the enhanced production of the glycolipid biosurfactant which is ideal for the industrial scale production and possible for the development of many industrial applications.

KEY WORDS

Candida tropicalis VEMS 27; Yeast; Biosurfactant production; Emulsification activity; Purification; TLC

1. INTRODUCTION

Surfactants are an important class of chemical compounds included in almost all of the everyday products we use. They can be found in detergents, laundry formulations, household cleaning products, cosmetics, herbicides, pesticides and also used for bioremediation, agriculture, food, pharmaceutical, textile, paper, petroleum industries, etc (1). Surfactants are amphiphilic molecules that tend to accumulate at the interfaces between fluid phases with different polarities (e.g., oil-water or air-water), reducing surface tension and increasing emulsification (2). Most of the surfactants currently used are chemically synthesized from petrochemical derivatives and are only partially biodegradable, causing detrimental effects to the environment (3).

Due to the increasing knowledge on environmental awareness, the demand for surfactants obtained from bio-based resources is increasing. Biosurfactants are surface-active compounds synthesized by a variety of microbes and are attracting a pronounced interest owing to their potential advantages over their chemical counterparts, mainly due to their lower toxicity and higher biodegradability (4). Biosurfactants display a wide variety of chemical structures, including glycolipids, lipopeptides, phospholipids, fatty acids, or neutral lipids, among others (5). *Bacillus* species produce a broad spectrum of biosurfactants, which are cyclic molecules consisting of a fatty acid of variable

length (hydrophobic moiety) linked to a short peptide chain (hydrophilic moiety) of seven or ten aminoacids.

As a consequence, biosurfactants can replace synthetic surfactants in many fields (6). However, the application of biosurfactants depends on whether they can be produced economically at large-scale. Presently, biosurfactants are not competitive with chemical surfactants from an economic point of view, since expensive substrates are required for their production and they are presently at low productive rates, which hamper their widespread use and commercialization (7). As the culture medium can account for up to 30–50% of the overall production costs of biosurfactants, choosing right choice of medium requirement at correct concentration for the enhanced production could be one among the best solution for reducing the cost of biosurfactant production. In this work, the enhanced production of biosurfactant was studied with a potential biosurfactant producing estuarine yeast, *Candida tropicalis* VEMS 27 using easily available lab chemicals and the produced biosurfactant was purified and biochemically characterized using standard procedures.

2. MATERIALS AND METHODS

2.1. Biosurfactant producing estuarine yeast and culture conditions

A biosurfactant producing estuarine yeast strain, *Candida tropicalis* VEMS 27 was used in this study for the enhanced biosurfactant production using different physicochemical parameters and studied for the basic biochemical characterization in its purified form. This strain was isolated from mangrove sediment samples of Vellar estuary, Porto Novo, Tamil Nadu, India and characterized as potential biosurfactant producer based on multiple screening methods viz., hemolytic activity, oil displacement test, BATH assay and emulsification index. Further, *C. tropicalis* VEMS 27 was identified based on 18S rRNA molecular sequencing method and the sequence was submitted to NCBS gene bank with the accession number, KT449837. Since the strain is from estuarine origin, it was maintained in 50% aged sea water prepared yeast malt (YM) agar (HIMEDIA, Catalogue No. M424) slants as axenic culture under refrigeration at 4°C. Further, monthly repeated culturing was done using the same medium under Petri plate conditions at 28°C and pH 6.5 for 48 h incubations to maintain its viability and purity.

2.2. Optimization of physicochemical parameters for enhanced biosurfactant production

The optimization was done by adopting search technique i.e., varying one parameter at a time. The experiments were conducted in 2 L Erlenmeyer flask using the mineral salt medium, Bushnell Haas broth (HIMEDIA, Catalogue No. M350) supplemented with 0.1% crude oil at 28°C and pH 6.5 as the basal media conditions and all the experiments were carried out in triplicate and the average values were calculated. During the optimization, the basal conditions were replaced with appropriate parameters and the value of a particular parameter achieved by one step was fixed in subsequent experiments. Factors like incubation period, carbon and nitrogen sources, pH, temperature and salinity were tested at different ranges. The enhanced biosurfactant production was determined with the cell free supernatant using 24 h emulsification activity (EA) assay (8).

2.2.1. Time course on production

Incubation was carried out for a time period ranging from 0 h to 72 h with 6 h interval in which the peak production time was estimated.

2.2.2. Carbon sources

The effect of various carbon sources like glucose, fructose, maltose, sucrose and starch at 2% substrate concentration was examined.

2.2.3. Nitrogen sources

The effect of different nitrogen sources like peptone, yeast extract, beef extract, tryptone and ammonium nitrate were prepared at 1% concentrations and tested for enhanced biosurfactant production.

2.2.4. pH

For the evaluation of optimum pH range, the culture medium was prepared in pH range from 5 to 9 with an interval of pH 1.

2.2.5. Temperature

The effect of temperature on biosurfactant production was tested from the range, 25˚C to 45˚C with an interval of 5˚C.

2.2.6. Salinity

Medium was prepared with ranges of salinity from 0ppt to 35ppt with an interval of 5ppt and inoculated to test the effect of salinity since the strain is from estuarine origin.

2.3. Isolation and purification of biosurfactant After optimal incubation, cultures were centrifuged at 3,000 rpm for 30min. and the cell free supernatant was subjected to acid precipitation using 6N HCl until pH 2

was attained (9). The treated broth was kept at 4°C overnight for precipitation of biosurfactant. Yellowish white precipitate was collected by centrifugation at 10,000 rpm for 30 min. The collected biosurfactant was then re-suspended in phosphate buffer at pH 7 and neutralized. The re-suspended residue was purified in a silica gel (60–120 mesh) column and the elutions were made with chloroform and methanol ranging from 20:1 to 2:1 v/v in a gradient manner and 10 fractions were obtained (10). The fractions were pooled, and the solvents were evaporated, the resulting residue was dialyzed against distilled water and lyophilized. The purified biosurfactant was analyzed for further studies.

2.4. Biochemical characterization

The extracted biosurfactant from the above procedure was characterized for its biochemical compounds present in it. The purified biosurfactant was biochemically determined using silica gel TLC plate which was separated using CH_3Cl: CH_3 OH: H_2O (65/15/2(v/v/v)) as the developing system. Visualizing reagents used were ninhydrin reagent (0.2 g ninhydrin in 100 mL ethanol) to detect peptides, anthrone reagent (1 g anthrone in 5 mL sulfuric acid mixed with 95 mL ethanol) to examine sugars and lipid portion was evidenced using rhodamine B reagent (0.25g in 100 ml ethanol).

3. RESULTS AND DISCUSSION

3.1. Optimization of physicochemical parameters for maximum biosurfactant production

Cell growth and production of metabolites were strongly influenced by optimizing its media composition such as carbon and nitrogen sources, salinity and other growth factors, thus optimizing media ingredients plays an important role in the high yield of biosurfactant (11). In this study, optimization of various physicochemical parameters was carried out with the potential estuarine yeast, *C. tropicalis* VEMS 27 using one factor at a time method.

3.1.1. Time course on biosurfactant production

The right time cultivation of the product plays an important role in maximizing product quantity. A sample of 10ml broth was withdrawn from cultured media regularly with 6 h interval from 0h to 72h and analyzed for emulsification activity (EA). The samples were aseptically taken, centrifuged and assayed immediately. The peak production was achieved after 42h (55.5% EA) of incubation and it was maintained up to 60h. Further, 42h incubation was taken for further analysis which is the shortest period of time conception as estimated (Fig. 1). A study has agreed our results that there was no significant difference observed in the yield of biosurfactant between 42 h and 84 h and suggested that the lipopeptide biosurfactant produced by the marine strain, *Aneurinibacillus aneurinilyticus* SBP-11 was growth dependent (12).

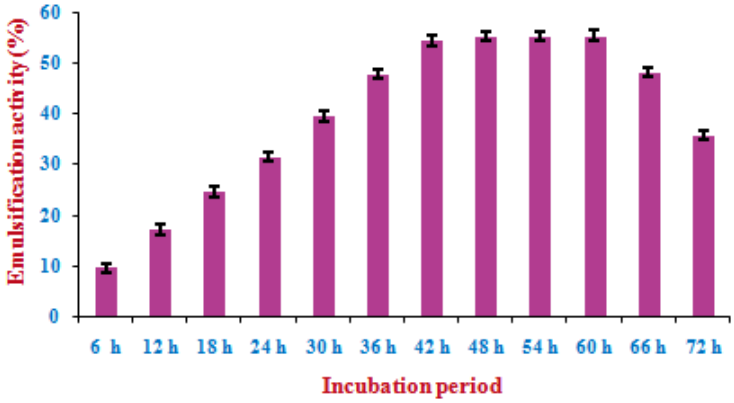

Fig. 1: Time course on biosurfactant production

3.1.2. Influence of different carbon sources on biosurfactant production

Numerous reports reveal that the type of carbon substrates markedly affected the production yield of biosurfactants (13, 14). The major types of carbon sources used for biosurfactant production were hydrocarbon and vegetable oils (13). In this work, five different nutritional carbon substrates were used, and the results showed that the strain, *C. tropicalis* VEMS 27 was able to grow in medium containing any of the five

sources. The performance of biosurfactant production with different carbon sources is depicted and reached a maximum product with 2% of glucose with 58.3% EA (Fig. 2) which is in good agreement with the results of Das and Mukherjee, (15) using *Bacillus subtilis.* Similar result was observed using glucose and glycerol were effective carbon substrates for rhamnolipid production with 136.4 and 71.8 mg/L/h with *Pseudomonas aeruginosa* (16).

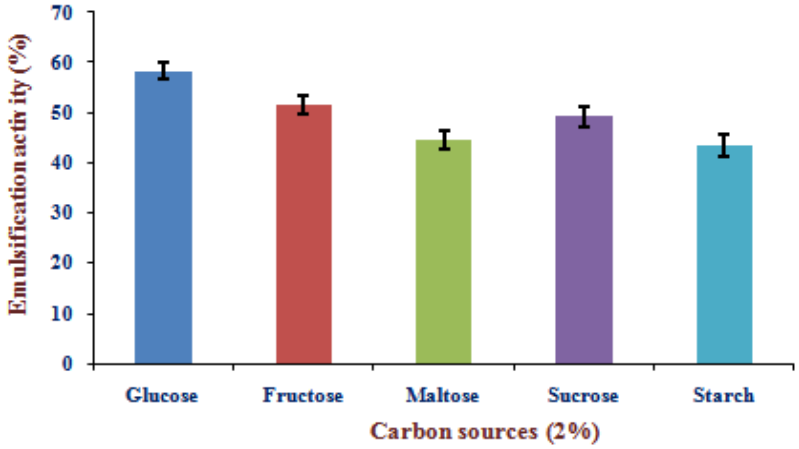

Fig. 2: Optimization of carbon sources for maximum biosurfactant production

3.1.3. Effect of various nitrogen source on biosurfactant production

Different nitrogen sources were estimated in this study for maximizing the production of biosurfactant. Higher level of nitrogen source influences the growth of the organism but not the production of the biosurfactant. Among the tested organic and inorganic nitrogen sources, ammonium nitrate increased the growth as well as showed highest yield of biosurfactant with 61.7% EA (Fig. 3). Contradictory to this, the yeast extract showed maximum growth and biosurfactant production of the potential strain, *Aspergillus ustus* MSF3 (17). Further, a study supported our report that the inorganic nitrogen source, sodium nitrate revealed ideal substrate for both microbial growth and rhamnolipid production using *Pseudomonas nitroreducens* (18).

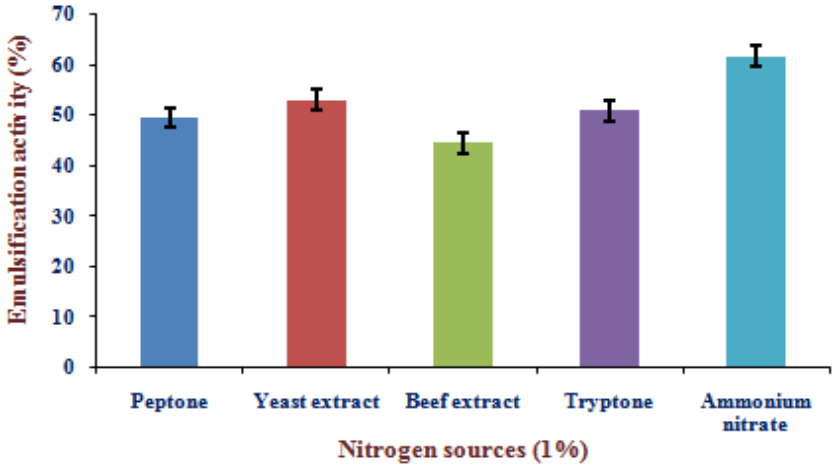

Fig. 3: Standardization of nitrogen sources for maximum biosurfactant production

3.1.4. Use of different pH on biosurfactant production

One of the important characteristics of most organisms is their strong dependence on pH for cell growth and production of metabolites. The strain, *C. tropicalis* VEMS 27 showed the highest biosurfactant production at pH 7.0 with 64.9% EA (Fig. 4). This observation has good argument with the results of biosurfactant production using the marine bacterium, *Bacillus simplex* which has showed maximum yield at pH 7 (19).

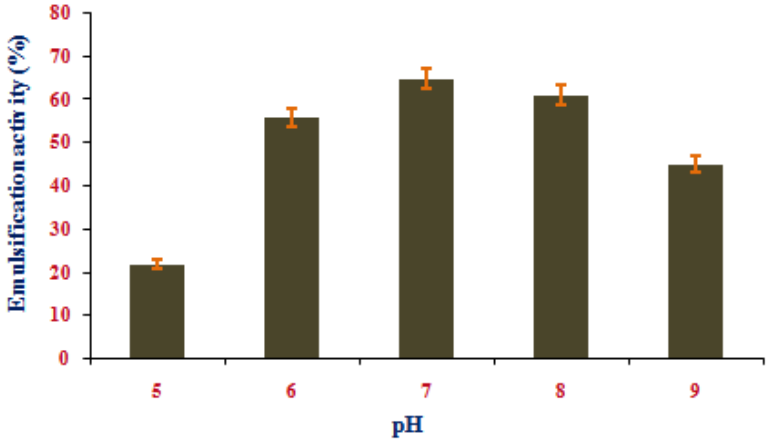

Fig. 4: Effect of different pH for maximum biosurfactant production

3.1.5. Optimization of various temperatures on biosurfactant production

Every microbial culture has its own optimum temperature which influences the growth as well as their metabolites production. To explore the effect of different temperatures on biosurfactant production with *C. tropicalis* VEMS 27, the strain was studied between 25 to 45˚C. The peak production was observed between 35 to 40˚C temperature with 67.9% EA (Fig. 5) and it was taken as 35˚C in view of energy conception. Further, *C. tropicalis* VEMS 27 was unable to grow at 45˚C, leading to decreased production of biosurfactant. Close to this, the optimal temperature for maximum rhamnolipid production by *P. aeruginosa* J4 strain was observed between 30 to 37˚C (20).

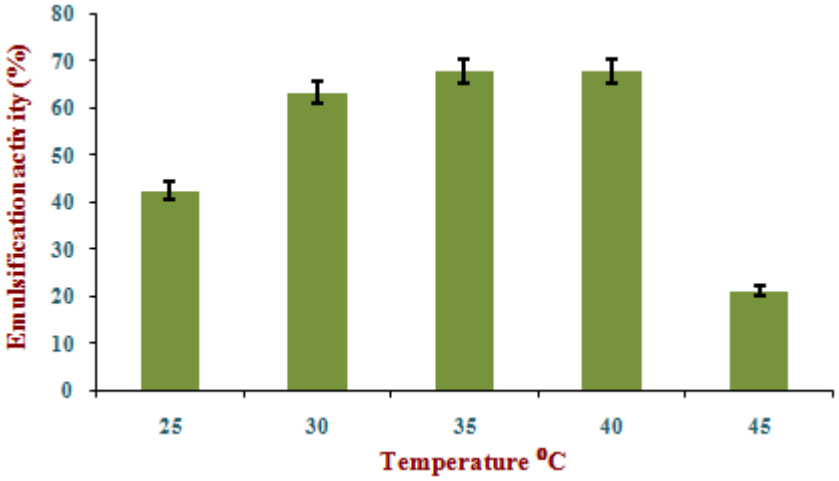

Fig. 5: Influence of different temperature for maximum biosurfactant production

3.1.6. Influence of different salinity on biosurfactant yield

Salinity is a major factor which significantly affects biological activity in the marine and estuarine environment (21) and the isolated *C. tropicalis* VEMS 27 is from estuarine origin, so the need of optimizing salinity makes an important estimation about the affinity of the organism to the salt concentration as well as its biosurfactant production. In this study, *C. tropicalis* VEMS 27 showed maximum biosurfactant production at 10ppt with 69.2% EA (Fig. 6). However, Kiran et al. (17) observed that salinity with 30ppt showed maximum biosurfactant production using *Aspergillus ustus* MSF3.

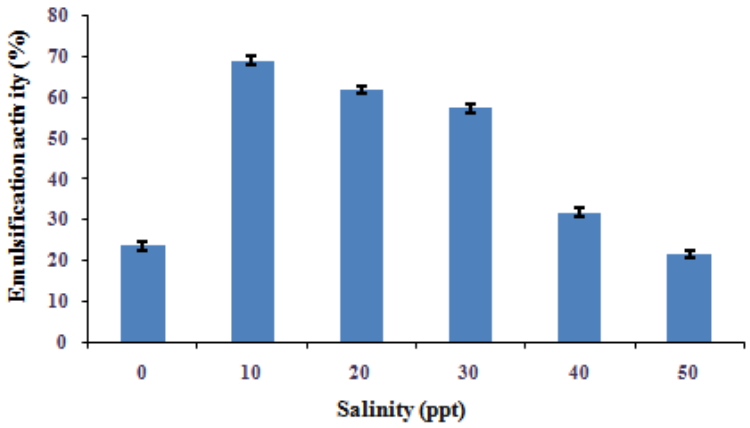

Fig. 6: Effect of different salinity for maximum biosurfactant production

3.2. Purification and biochemical characterization of biosurfactant

Using the optimized growth conditions, the biosurfactant was produced and isolated by centrifugation followed by acid precipitation procedures. The precipitate was subjected for purification using silica gel column chromatography with different solvent ratios of methanol and chloroform. Ten fractions were made and each fraction was studied for emulsification activity and found that the purified biosurfactant was eluted from 4:1 to 2:1 fractions. The eluted biosurfactants were rotary evaporated and dried which showed the dry weight concentration of 4.85g/L. Further, the purified biosurfactant was qualitative assessed for its biochemical composition using thin layer chromatography which has showed the presence of two biochemical compound viz., lipid and carbohydrate on TLC plates with Rf value 0.73 and 0.45 (Fig. 7). These results clearly suggesting that the biosurfactant has fatty acid and carbohydrate content and protein was found to be absent which representing it is a biosurfactant of glycolipid nature. Similar to this, the biosurfactant produced by marine *Staphylococcus saprophyticus* SBPS 15 has the combination of carbohydrate and lipid compounds using TLC method and confirmed it has the glycolipid nature (22).

4. CONCLUSION

The present study investigated medium optimization using the estuarine yeast, *C. tropicalis* VEMS 27 which revealed significant response to the adopted various physicochemical parameters for the enhanced biosurfactant production and the produced biosurfactant was purified using a simple chromatographic technique. These observations proved the possibilities for the industrial scale production of biosurfactant and further application studies in future.

5. ACKNOWLEDGEMENT

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