ORIGINAL ARTICLE

Substrate Optimization Using 0.25g substrate of Molasses to produce Biosurfactant by *Pseudomonas aeruginosa*

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ABSTRACT

Aim: To produce biosurfactants from Pseudomonas aeruginosaon substrate optimization of 0.25g using agricultural resource and to produce Biosurfactants using low cost materials.

Study design: Observational Descriptive study

Place and duration of study: Study was conducted at Institute of molecular biology and biotechnology in university of Lahore. Duration of the study was two years.

Methods: The volume of sample taken 0.25g, of inoculum from growing culture of *Pseudomonas aeruginosa* was isolated and collected from industrial area of District Kasoor and flasks were than placed into an orbital shaker at speed of 120rpm. The samples were collected in sterile screw capped bottle, aseptically. The samples were stored at 4°C till further use.

Results:-. Surface tension was 53.5, 47.7, 34.5 and 30.8mN/m at time 24, 48, 72 and 96 hours respectively at constant temperature of 37°C and molasses used 0.25g with 1ml inoculum size. The rhamnolipid production was 0.42, 1.95, 2.89 and 3.09 g/L respectively. Similarly the bacterial cell mass was 0.1, 0.5, 1.0 and 1.6 g/L respectively.

Conclusion: After optimizing various growth and environmental factors a production of rhamnolipid was achieved.

Keywords: Pseudomonas aerouginosa, biosurfactant

INTRODUCTION

Surfactants can be derived from both petrochemical feedstock and renewable resources e.g., plant and animal oils, micro-organisms. They were originally made from renewable resources like fats and oils, whereas today, the majority is of petrochemical origin[1]. At present biosurfactants are unable to compete with the chemical surfactants due to their high production costs. As biosurfactants are readily biodegradable and can be produced from renewable and cheaper substrates, they might be able to replace their chemically synthesized counter parts[2]. Biosurfactants are useful as antibacterial, antifungal and antiviral agents. In addition, they also have potential for use as major immunomodulatory molecules, adhesive agents and even in vaccines and gene therapy. Most of the biosurfactants used in biological applications are required in very low concentrations that make biosurfactants valuable biomolecules for applications as food additives, specialty chemicals, biocontrol agents, and new generation molecules for health and beauty care industries[3]. Molasses is a by-product of the sugar industry that is low in price compared to other conventional sugar sources like sucrose or glucose and is rich in other nutrients such as minerals and vitamins. Two *B. subtilis* strains were able to produce lipopeptide surfactants using minimal medium supplemented with molasses as carbon source[4]. Molasses and corn steep liquor were used as the primary carbon and nitrogen sources for production of rhamnolipid biosurfactants by *P. aeruginosa* GS3; the interfacial tension of culture medium against crude oil was reduced from 21 to 0.47 mN/m[5]. The present work was designed to use Molasses as a carbon source for the production of biosurfactants and to develop a cheaper method to produce such useful molecules by fermentation process. The objectives of our study were to: to produce Biosurfactants using low cost materials. And to develop a Production Process based on optimization of media component and environmental factors.

MATERIALS & METHODS

It was designed to optimize the inoculum size for the production of rhamnolipid. The volume of sample taken are 1ml, of inoculum from growing culture of *Pseudomonas aeruginosa*was isolated from contaminated soil collected from industrial area of District Kasoor and flasks were than placed into an orbital shaker at speed of 120rpm. The samples were

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collected in sterile screw capped bottle, 4-5cm deep from the soil surface aseptically. The samples were stored at 4°C till further use[6]. After every 24h the culture broth from each flask was taken to estimate bacterial cell mass. All the chemicals including L-rhamnose, Orcinol reagent, Diethyl ether, Molasses, Na_2HPO_4, K_2HPO, $MgSO_4$, NaH_2PO_4, $FeSO_4$, Peptone were purchased from Sigma Aldrich from their local distributor in Lahore, Pakistan. The bacterial strains were isolated from the industrial contaminated soil by using soil enrichment technique. Briefly; 1g soil from sample, in 100ml sterile mineral salt media with 1g of molasses was incubated for 96 hours at 37°C on an orbital shaker at 100 revolutions per minute. After enrichment, cell suspension was taken from the flask and spread over nutrient agar plate and was incubated at 30°C for 48 hours. Colonies that appear on nutrient agar plates were selected randomly and sub –cultured to obtain pure isolates[6]. An organic nitrogen medium, with phosphate was prepared. The composition of the medium was (gL^{-1}): NaH_2PO_4 .H_2O, 4.0, Na_2HPO_4 .H_2O, 1.0, $MgSO_4$.$7H_2O$, 1.0, $CaCl_2$.$2H_2O$, 0.005, Peptone, 1.38, 25ml of glycerol was used as source of carbon substrate[5]. A total of 2.5litres of distilled water was used, hence the above measured weights and volume respectively was calculated based on that. The pH of the medium was adjusted to 7 using 211 Microprocessor pH meter with 1.0M NaOH. Sixteen Erlenmeyer flasks (250ml) were used during the experiment. 150ml of the prepared medium was measured into each flask using a 200ml measuring cylinder. Each flask was clogged using cushion foam and covered with Aluminium foil. The prepared medium was autocleaved for 3 days before being inoculated. Nutrient broth media (100ml) was inoculated with bacterial strain and growth was monitored at 37°C in shaking incubator at 100 rpm for 72 hours[7].

RESULTS

Various carbon sources was used for the production of rhamnolipid biosurfactans by *Pseudomonas* species, as it is reflected from the literature that biosurfactant production is strongly influenced by the nature of carbon substrate[8]. The substrate used here was Molasses. Molasses contains high amount of sugars and have the potential to be used for the production of rhamnolipid. There are two types of molasses available on industrial scale sugar cane molasses and sugar beet molasses

Sugar beet molasses is about 50% sugar by dry weight, predominantly sucrose but also containing significant amounts glucose and fructose. The non sugar contents are calcium, potassium, oxalate and chloride. Sugar cane and sugar beet molasses both produced in the huge quantities as a byproduct of sugar industry. Articles has been traced for the production of rhamnolipid using molasses[5]. It was found that molasses is one of the best source for the production of rhamnolipid biosurfactant as 3.09g/L of rhamnolipid was estimated after 96h of incubation using 1ml of inoculum size per 100ml of broth. Biosurfactant production was previously studied using medium with varying concentration of molasses being used as the sole source of carbon[5]. 0.25g concentrations was used and added into the fermentation media. The experiment was observed for 96h and temperature was maintained at 37°C at 7 pH. Flasks containing varying concentrations of molasses with 1ml inoculum size i.e. *Pseudomonas aeruginosa* were placed into an orbital shaker at speed of 120 rpm.. After every 24h the culture broth from each flask was taken to estimate bacterial cell mass, rhamnolipid estimation and surface tension reduction. This study was designed to optimize the substrate size and substrate sizes of 0.25g,is used. Results produced by this substrate sizes is summarized below in (Table 1).

The results of the present study (Table 1) revealed that surface tension was 53.5, 47.7, 34.5 and 30.8mN/m at time 24, 48, 72 and 96 hours respectively at constant temperature of 37°C and molasses used 0.25g with 1ml inoculum size. The rhamnolipid production was 0.42, 1.95, 2.89 and 3.09 g/L respectively. Similarly the bacterial cell mass was 0.1, 0.5, 1.0 and 1.6 g/L respectively. Biosurfactant Production is a growth associated production; parallel relationships exist between growth, substrate utilization and biosurfactant production. The production of rhamnolipid by *Pseudomonas* species is an example of growth associated biosurfactant production[8]. It had been found that the best substrate size to obtain optimum microbial growth was 0.25g/100ml of broth. It was observed that as the substrate size increased the bacterial cell mass did not increased. Hence, showed that the initial concentration of the substrate should be 0.25g/100ml of broth (Fig. 1).

Table 1: Results with 0.25g substrate

Time	Inoculum size ml	Temp.°C	Molases g	Surface Tensionm N/m	Rhamnolipidg/L	Bacterial cell massg/L
24 hrs	1	37	0.25	53.5	0.42	0.1
48 hrs	1	37	0.25	47.7	1.95	0.5
72 hrs	1	37	0.25	34.5	2.89	1.0
96 hrs	1	37	0.25	30.8	3.09	1.6

Fig. 1: Estimation of Bacterial cell mass using 0.25g substrate

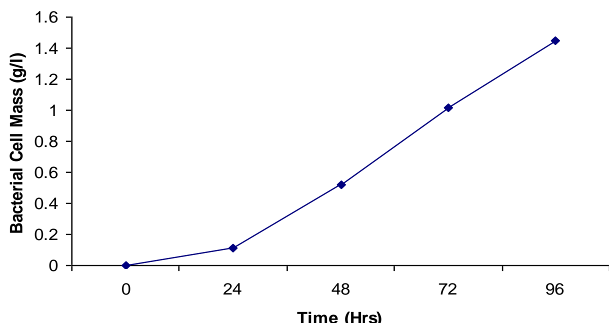
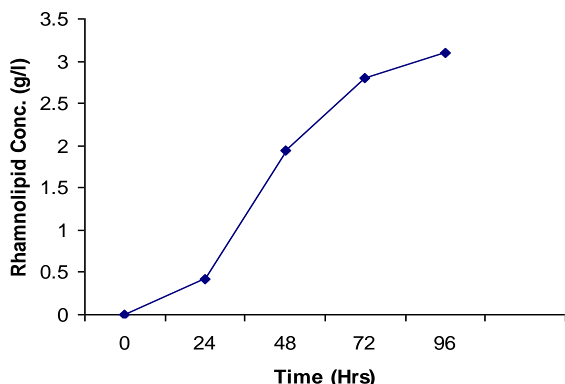


Fig. 2: Estimation of Rhamnolipid production using 0.25g substrate



The Fig. 1 represents that bacterial cell mass (g/L) increased with the passage of time as revealed in the fig that at zero time the bacterial cell mass was zero and it increased to 1.6 g/L bacterial cell mass when the time passage was 96 hours. The effect of substrate concentration was also determined by rhamnolipid production, as it is shown by the results that 0.25g substrate per 100ml of broth can produce 3.09g/L after 96h. In contrast high sugar concentration has not much effect on rhamnolipid production (Fig. 2).

The Fig. 2 represents that rhamnolipid concentration (g/L) increased with the passage of time as revealed in the fig that at zero time the rhamnolipid concentration was zero and it increased to 3.09 g/L rhamnolipid concentration when the time passage was 96 hours.

DISCUSSION

Substrate optimization is very important factor for microbial production. As molasses was used which is sugar so it is very important to reveal the desired molasses concentration in which bacteria can survive, as high salt and sugar concentrations leads to the loss of water from cell and ultimately bacterial cell death[8]. Substrate size 0.25g/100ml of broth were tested and rhamnolipid production, surface tension and bacterial cell mass was estimated. Our results showed that the production achieved with this research was 3.09g/L by using 0.25g molasses which was not as much good as previously achieved but this yield can be increased by further optimizing growth and environmental factors. The production of rhamnolipids by *Pseudomonas aeruginosa*using different quantities of substrate as n-hexadecane, molasses and glycerol was 6g/L)[6]. Rhamnolipid production is a growth associated process[8]. The substrate optimization in media was very important parameter as stated above that high sugar concentration can kill bacterial cells.

CONCLUSION

After optimizing various growth and environmental factors a production of rhamnolipid was achieved.

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