ORIGINAL ARTICLE

Biosurfactant Production by *Pseudomonas aeruginosa* Strains on 3ml of inoculum size

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ABSTRACT

Aim: To produce biosurfactants from Pseudomonas aeruginosa using agricultural resource and to produce Biosurfactants using low cost materials.
Study design: 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 are 3ml, of innoculum from growing culture of *Pseudomonas aeruginosa* was isolated from contaminated soil collected from industrial area of District Kasoor and flasks were then placed into an orbital shaker at speed of 120rpm. The samples were collected in sterile screw capped bottle, 4-5cm deep from soil surface aseptically. Samples were stored at 4^oC till further use. After every 24h, culture broth from each flask was taken to estimate bacterial cell mass.
Results: Surface tension was 66.1, 63.2, 48.2 and 45.7 mN/m at time 24, 48, 72 and 96 hours respectively at constant temperature of 37°C and molasses used 0.25g with 3ml inoculum size. The rhamnolipid production was 0.23, 0.82, 1.75 and 1.98g/L respectively. Similarly the bacterial cell mass was 0.2, 0.18, 0.22 and 0.4 g/L respectively.
Conclusion: After optimizing various growth and environmental factors a production of rhamnolipid was achieved.
Keywords: Biosurfactant, pseudomonas aeruginosa, inoculum

INTRODUCTION

The amphiphiles that form micelles and can be potentially used for surface chemical works are termed surface active agents or surfactants. The enormous market demand for surfactants is currently met by numerous synthetic, mainly petroleum-based chemical surfactants. These compounds are usually toxic to the environment as well as non-biodegradable. They may bio-accumulate and their production, processes and by-products can be environmentally hazardous. It has become important that tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternates to chemical surfactants[1].

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The worldwide production of surfactants amounted to 17million metric tonnes (t) in year 2000 (including soaps), with expected future growth rates of 4% per year globally and 2% in the Europe[2]. In recent years greater emphasis has been placed on the environmental impacts of chemical surfactants and new surfactants for use in the pharmaceutical and biomedical. For example, a range of new nonionic gemini aldonamide-type surfactants consisting of two hydrophobic chains and two aldonamide polar head groups fused with a linker region have been developed that have low critical micelle concentration values (3.8×10^{-6} to 1.3×10^{-4} M)[3]. Regarding the environmental impact and toxicity of the chemical surfactants interest in the biological surfactants is steadily increasing. Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi[4] from various substances including sugars, oils and wastes. However, carbohydrates and vegetable oils are among the most widely used substrates for research on biosurfactant production by *Pseudomonas aeruginosa* strains[4].

Bacteria of the genus *Pseudomonas* are known to produce glycolipid surfactant containing rhamnose and 3-hydroxy fatty acids[5,7].

Rhamnolipids produced by *Pseudomonas aeruginosa* have been widely studied and reported as a mixture of homologous species RL1 ($RhC_{10}C_{10}$), RL2 (RhC_{10}), RL3 ($Rh_2C_{10}C_{10}$) and RL4 (Rh_2C_{10})[6,7]. From a combined application/cost perspective rhamnolipid, produced by *P. aeruginosa*, represents the leading commercial microbial biosurfactant and hence this brief discourse on industrial biosurfactant production will be confined to this product/host system. Extensive investigations have been implemented at both the molecular and cell culture level aimed at understanding factors influencing rhamnolipid biosurfactant biosynthesis by *P. aeruginosa* with a view to optimising the fermentation process[8]. Molasses: Molasses is a co-product of sugar production, both from sugar cane as well as from sugar beet. It is defined as the runoff syrup from the final stage of crystallization, in which further crystallization of sugar is uneconomical. Molasses generally consists of 48-56% total sugar (mainly sucrose), 9-12% non-sugar organic matter, 2-4% protein (N×6.25), 1.5-5% potassium, 0.4-0.8% calcium, 0.06% magnesium, 0.6-2.0% phophorus, 1.0-3.0mg/kg biotin, 15-55 mg/kg pantothenic acid, 2500-6000 mg/kg inositol and 1.8 mg/kg thiamine[8]. Different kinds of bacteria have been employed by many researchers in producing biosurfactant using culture media. Most of such bacteria used are isolated from contaminated sites usually containing petroleum hydrocarbons by-products and/or industrial wastes[9].

MATERIALS AND METHODS

It was designed to optimize the inoculum size for the production of rhamnolipid. The volume of sample taken are 3ml, 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 collected in sterile screw capped bottle, 4-5cm deep from the soil surface aseptically. The samples were stored at 4^oC till further use[10]. 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^oC on an orbital shaker at 100 revolutions per minute. After enrichment, 3ml cell suspension was taken from the flask and spread over nutrient agar plate and was incubated at 30^oC for 48 hours. Colonies that appear on nutrient agar plates were selected randomly and sub–cultured to obtain pure isolates[10]. 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 (11). 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 3days before being inoculated. Nutrient broth media (100ml) was inoculated with bacterial strain and growth was monitered at 37^oC in shaking incubator at 100 rpm for 72 hours[12].

RESULTS

It was designed to optimize the inoculum size for the production of rhamnolipid. Various volumes of inoculum were taken and added into the fermentation media. The experiment was monitored for 96hours and temperature was set at 37°C and pH was set at 7. The volumes taken 3ml of inoculum from growing culture of *Pseudomonas aeruginosa* and flasks were than placed into an orbital shaker at speed of 120rpm. After every 24h the culture broth from each flask was taken to estimate bacterial cell mass, rhamnolipid estimation and surface tension reduction. (Tables 1).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[13].

Table 1: Results with 3 ml inoculum size

No	Time hours	Inoculum size ml	Temp.°C	Molases g	Surface Tension mN/m	Rhamnolipids g/L	Bacterial cell mass g/l
9	24	3	37	0.25	66.1	0.23	0.2
10	48	3	37	0.25	63.2	0.82	0.18
11	72	3	37	0.25	48.2	1.75	0.22
12	96	3	37	0.25	45.7	1.98	0.4

The results of the present study (Table 1) revealed that surface tension was 66.1, 63.2, 48.2 and 45.7 mN/m at time 24, 48, 72 and 96 hours respectively at constant temperature of 37°C and molasses used 0.25g with 3ml inoculum size. The rhamnolipid production (Fig 2) was 0.23, 0.82, 1.75 and 1.98 g/L respectively. Similarly the bacterial cell mass (Fig 1) was 0.2, 0.18, 0.22 and 0.4 g/L respectively.

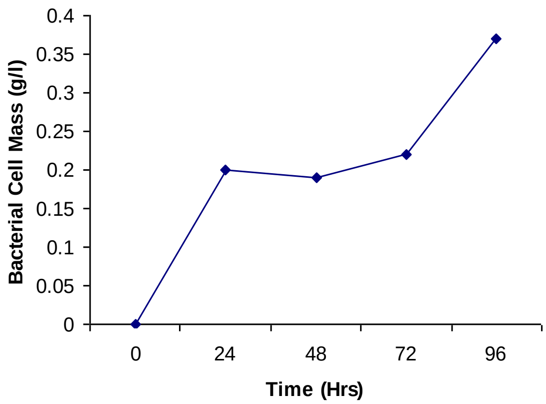

Fig. 1:Estimation of bacterial cell mass using 3ml inoculum size

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 0.4g/L bacterial cell mass when the time passage was 96 hours.

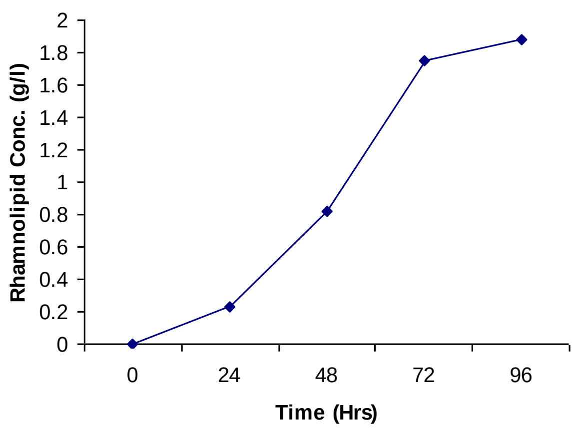

Fig. 2: Estimation of rhamnolipid production using 3ml inoculum size

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 1.98g/L rhamnolipid concentration when the time passage was 96 hours.

DISCUSSION

Inoculum size is one of the most important parameter for the production of microbial metabolites. Microorganisms required a certain cell number in a particular media to start their rapid growth and metabolite production (log phase) so it is important to determine the exact initial bacterial size to start an experiment leading to the successful end. As rhamnolipid is a growtn associated process[13,14], the optimization of the inoculum size in proposed media was the most important parameter to be optimized. It was found that 1ml inoculum size was best for rhamnolipid production during this research. it was shown by that biosurfactant production was growth associated so increase in inoculum size will increase the nutritional demand by microorganisms[13,14] so it was very essential for the experiment to maintain a balance between the inoculum size and the volume of the media component as it effected the biosurfactant production shown by the results. 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[13,14].

CONCLUSIONS

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

Acknowledgments: The authors highly acknowledge the honourable dean Dr Saghir Ahmad Jafri, Dean, Faculty of Sciences, Director, Mr. Asif Jamal, The University of Lahore.

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