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Central European Journal of Experimental
Biology, 2012, 1 (1):26-35
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Estimation of Biosurfactant Activity of an Alkaline Protease Producing Bacteria Isolated from Municipal Solid Waste

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ABSTRACT:

Biosurfactants are surface-active substances synthesized by microorganisms having the properties of reducing surface tension, stabilizing emulsions, promoting foaming and are generally non-toxic and biodegradable. Here an effort was made to screen biosurfactant activity of a protease producing bacteria isolated from municipal solid waste. Strain was identified as *Pseudomonas aeruginosa* by 16S rDNA based molecular technique. Biosurfactant, obtained from isolated organism was screened by hemolytic assay, drop collapsing method, oil spread method, blue agar plate method and oil spreading technique. In oil spread method kerosene oil shown the best result (58mm) and in emulsification index method petrol has given maximum efficient result (64 % in comparison to 1% of SLS having 58% EI). Besides biosurfactant activity the strain also produces protease enzyme. The strain has shown maximum protease activity at pH 9.5, temperature 37°C and 48 hrs. of incubation time. So, this strain can be used in textile, leather, detergent, pharmaceutical and dairy industries for its dual ability of producing protease enzyme and biosurfactant activity.

Key words: Biosurfactant, *Pseudomonas aeruginosa*, 16S rDNA based molecular technique.

INTRODUCTION

Recently, the products of biological origin, particularly enzymes, are attracting the attention of the researchers. Among the enzymes, proteases occupy an important position as they were the first to be produced in bulk, and now constitutes ~ 66% of total enzymes employed [1]. Proteases are present in all living organisms. But microbial proteases are most exploited group of industrial enzymes. Based on the mode of action, they are further classified into four categories viz. alkaline, acid, thiol and metallo proteases [2]. Since alkaline proteases are active over a broad pH (7-12) and temperature (35°C-80°C) ranges [3].

Protease is applied in pharmaceutical, food and detergent industries, waste treatment and others. Biotechnological importance of these enzymes has been realized by leather industries for the purpose of dehairing and bating hides as a substitute toxic chemical [4]. In food industry proteases are used as crude preparation. In pharmaceutical industry they are used as ingredients of ointments for debriment of wards and in medicine preparation [5].

Biosurfactants are amphiphilic biological compounds produced extracellularly or as part of the cell membrane by a variety of yeast, bacteria and filamentous fungi [6]. Unlike synthetic surfactants, microbially-produced compounds are easily biodegradable and suited for bioremediation and dispersion of oil spills. Potential application of biosurfactants includes emulsification, wetting, foaming and surface activity that can be exploited in food, oil, cosmetic and pharmaceutical industries [7] and also possess antimicrobial activity against *Mycobacterium*

tuberculosis [8]. The total surfactant production has exceeded 2.5 million tons in 2002 for many purposes such as polymers, lubricants and solvents [9]. The present study was designed to isolate a protease producing bacteria from municipal solid waste as it is a rich source of many important bacteria [10] and screening for its biosurfactant activity. Here we have optimized the parameters for maximum protease and biosurfactant production. So that with these information specific future applications like as antitumor activity of this biosurfactant can be achieved [11].

MATERIALS AND METHODS

Source: Solid waste collected from Municipal Waste Dumping Yard, Kolkata.

Isolation and Screening: 1 gm of waste was suspended in 10 ml of sterile water and serial dilution 10^{-7} was selected and streaked on nutrient agar plate and incubated for 48 hrs at 37°C. Colonies were randomly selected and streaked on Skimmed Milk Agar plate to isolate protease producing bacteria. Two colonies produced clear zone on SMA plate and colony having higher zone was selected for experiment. Two screening broth such as: casein-yeast extract-lactose-mineral salt broth (casein-1.25%, yeast extract-0.5%, K_2HPO_4 -0.05%, $CaCl_2$ -0.05%, $MgSO_4$ -0.05%, Lactose- 0.05%) and tryptone-yeast extract-dextrose broth (tryptone-1%, dextrose-0.1%, yeast extract-0.5%) were used for final selection.

Biomass yield: Biomass yield was determined by measuring the absorbance at 600nm [12].

Enzyme assay: Tryptone-yeast extract-dextrose broth was selected as assay medium. Enzyme assay was determined by the modified method of Hayashi *et al.* [13].

As followed by Meyers and Ahearn [14]. Protease concentrations in samples were calculated from standard curves prepared with known concentration of tyrosine at 650 nm wavelength and expressed in Unit. One unit of enzyme was defined as the amount of enzyme that releases 1 μ g of tyrosine ml^{-1} of crude extract h^{-1} .

Optimization of culture conditions (for protease):

Effect of carbon source and concentration: Keeping the rest of the compositions and concentrations same of the assay medium, firstly the carbon sources were varied viz. dextrose, lactose, maltose, mannitol and sucrose and next the optimum carbon source concentration was varied as 0.025, 0.05, 0.075, 0.100, 0.125, 0.150 gm/100ml respectively. In both the cases 80 ml of media were taken in conical flasks and autoclaved at 121°C and 15 psi for 15 minutes. Equal amount of inoculums were added and incubated at $37 \pm 4^\circ C$ in BOD shaker incubator at 200 rpm.

Effect of protein source and concentration: The nitrogen source and optimum nitrogen source concentration were varied in the assay medium keeping the other compositions unchanged. The nitrogen sources used were tryptone, peptone, NH_4Cl and concentrations of optimum source were 0.50, 0.75, 1.00, 1.25, 1.50 gm/100 ml respectively.

Effect of medium pH: The effect of medium pH was determined by adjusting the medium pH ranging from 5.0 to 10.0 (viz. 5.0, 6.0, 7.0, 8.0, 9.0, 9.5, 10 respectively) using 1N HCl and 1N NaOH.

Effect of incubation temperature: The culture medium was incubated at different temperatures such as, $25 \pm 4^\circ$, $30 \pm 4^\circ$, $37 \pm 4^\circ$, $40 \pm 4^\circ$, $45 \pm 4^\circ C$ for optimum enzyme production.

Effect of incubation period: This effect period was studied by incubating the culture medium for different time period viz. 24, 48, 72, 96 hrs at $37 \pm 4^\circ C$

Screening for Biosurfactant Activity: Biosurfactant activity was screened by the following methods.

Drop Collapsing Method: Different oils (diesel, kerosene, castor oil, paraffin oil) in 5 μ l was added in 96- well microtitre plate and incubated at 37°C for 1hr. Culture supernatant (10 μ l) was added to the surface of the oil in the well. The shape of the oil surface was noted after 1 min. The collapsing of oil drop was indicated as positive result. Distilled water was used as negative control [15].

Hemolytic activity: Hemolytic activity was tested on blood agar plate. Plate was prepared with human blood (5%) and blood agar base and it was sterilized by autoclaving. Prior to pouring, blood was added. The strain was streaked on the plate and incubated at 37°C for 48 hrs [16].

Oil Spreading Technique: 50 ml of distilled water was added to large petridish followed by addition of 20 μ l of the above oils to the surface of water and 10 μ l of supernatant of 24 hrs incubated culture broth [17].

Emulsification Stability (E24) Test: E24 of culture sample was determined by adding 2 ml of each oils to the same amount of culture, mixing with vortex for 2 min and allowed to stand for 24 hrs. The E24 index is given as percentage of height of emulsified layer (mm) divided by total height of liquid column [18].

Blue Agar Plate (BAP) Method: Anionic surfactants, specifically rhamnolipids were detected by this technique. Mineral salt agar medium supplemented with cetyltrimethylammoniumbromide (CTAB- 0.2 gm/lit) - methylene blue (MB- 0.005gm/lit) was prepared along with carbon source as lactose (2%) [19]. A dark blue halo around the culture was considered as positive for biosurfactant production.

Biosurfactant Assay: Mineral salt medium, used for Blue Agar Plate (as assay medium) along with supplementation of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (10 mg/liter) with pH 6.7. From the above screening it was confirmed that the produced biosurfactant was rhamnolipid and the concentration of extracellular glycolipid was evaluated in triplicate by measuring the concentration of rhamnose with a modified orcinol method [20,21]. Rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose at 421 nm wavelength and expressed as rhamnose equivalents (in milligrams per millilitre) [22].

Optimization of culture conditions (for biosurfactant):

Effect of carbon source and concentration: Carbon sources were varied with same concentration viz. dextrose, lactose, mannitol and mannose and thereafter optimum carbon source was incorporated in different concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 gm/100ml respectively). In both cases 80 ml of media were taken in conical flasks along with equal amount of inoculums and incubated at $37 \pm 4^\circ\text{C}$ in BOD shaker incubator at 150 rpm.

Effect of protein source and concentration: Nitrogen sources used were yeast, peptone, NH_4Cl and tryptone and optimum nitrogen source was then varied, in different concentrations (0.09, 0.138, 0.190, 0.240, 0.290 gm/100 ml respectively) keeping the rest of the compositions constant.

Effect of FeSO_4 concentration: Only FeSO_4 concentration was varied in the assay medium to study its effect on bacterial growth and optimize biosurfactant production.

Effect of medium pH: The effect of pH was determined by adjusting the pH from 5.0 to 11.0 (viz. 5.0, 6.0, 7.0, 8.0, 9.0, 10 and 11 respectively) using 1N HCl and 1N NaOH.

Effect of incubation temperature: Culture media along with inoculum and adjusted pH were incubated at several temperatures ($30 \pm 4^\circ$, $37 \pm 4^\circ$, $40 \pm 4^\circ$, $45 \pm 4^\circ\text{C}$) for optimum biosurfactant production.

Effect of incubation period: The effect of incubation period was studied by incubating the culture medium for different time period viz. 24, 48, 72, 96 hrs at $37 \pm 4^\circ\text{C}$.

RESULTS

Although proteases are wide spread in nature, microbes serve as preferred source of these enzymes because of their rapid growth, limited space required for their cultivation and separation. The microbes can also be genetically modified to generate new enzymes with altered properties and very high yield, which are desirable for their various applications [23]. The isolated strain showed better protease production (activity) in casein-yeast extract-lactose-mineral salt medium and selected for further studies. (Fig.1)



Fig.1: Skimmed Milk hydrolyzed by *Pseudomonas aeruginosa*

Identification of Isolated Strain: Isolated strain was identified as *Pseudomonas aeruginosa* from Xcelris Labs Ltd. (Sydney House, Ahmedabad, India) using 16S rDNA based molecular technique which is available online data base of NCBI having GenBank accession number- bankit1363890 HM637743.

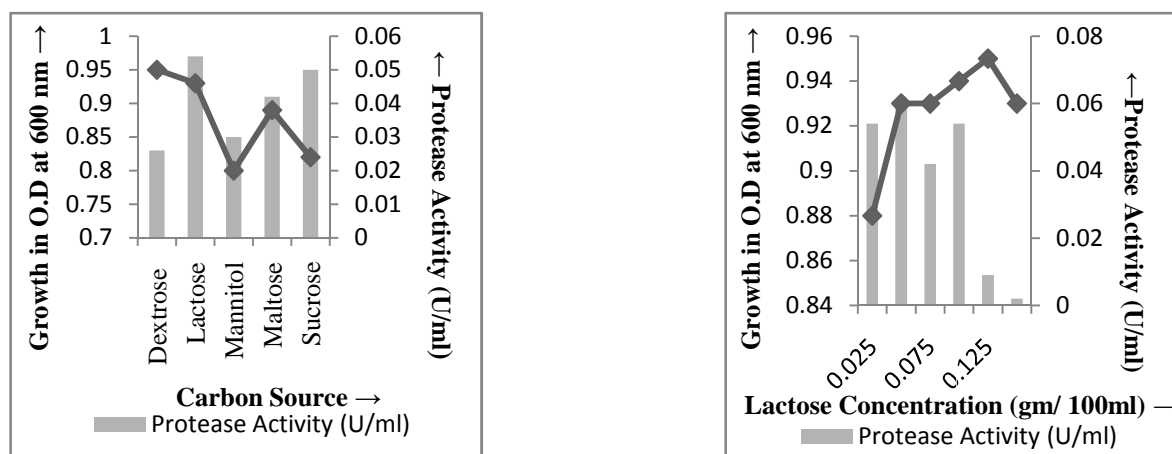


Fig.2: Comparison of bacterial growth and protease activity on carbon source and Concentration

Effect of protein source and its concentration: Different protein sources had been studied and maximum protease production was observed with casein and with concentration of 1.25 gm/100 ml. (Fig.3)

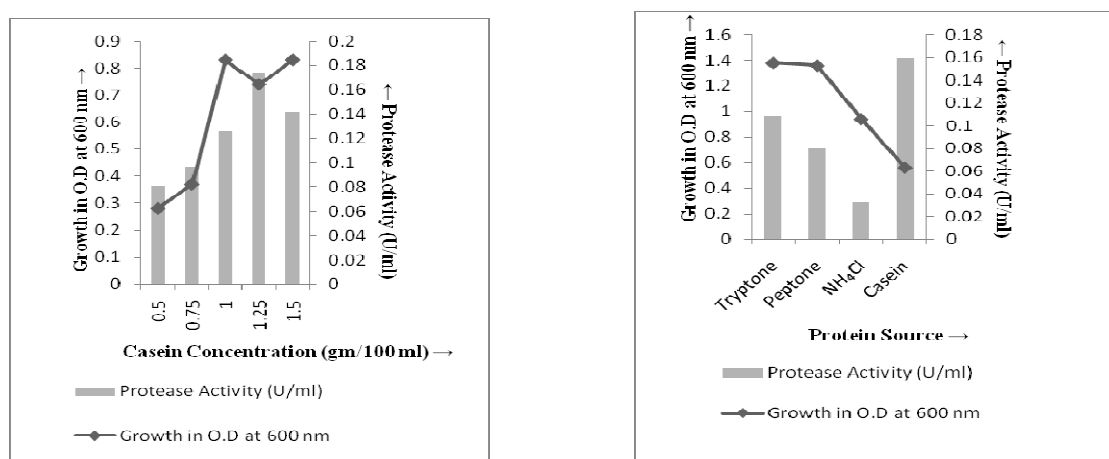


Fig.3: Comparison of bacterial growth and protease activity on protein source and concentration.

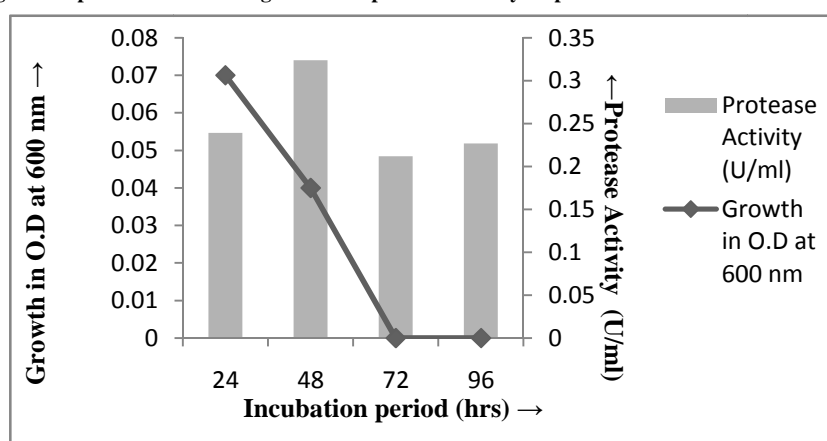


Fig.4: Comparison of bacterial growth and protease activity depending on incubation period.

Optimization of culture conditions (for protease enzyme):

Effect of carbon source and its concentration: Study showed that lactose was the most favorable carbon source and 0.05 gm/100 ml concentration produced maximum protease production (Fig.2). Similar results have been

reported by Malathi and Chakraborty(1991) that lactose was the best carbon source for enzyme production by *Aspergillus flavus* [24].

Effect of incubation period: When the bacteria was grown on casein-yeast extract-lactose-mineral salt medium it showed highest enzyme activity at 48 hrs incubation period which was less in further 72 and 96 hrs incubation (Fig.4). It shows that protease production was independent on the growth of the bacterium.

Effect of medium pH: Microorganisms are very sensitive towards medium pH for their growth and enzyme activity. So, optimum pH was investigated and growth characteristic and enzyme activity was recorded. The highest growth and enzyme activity was found at pH 9.5(Fig.5).

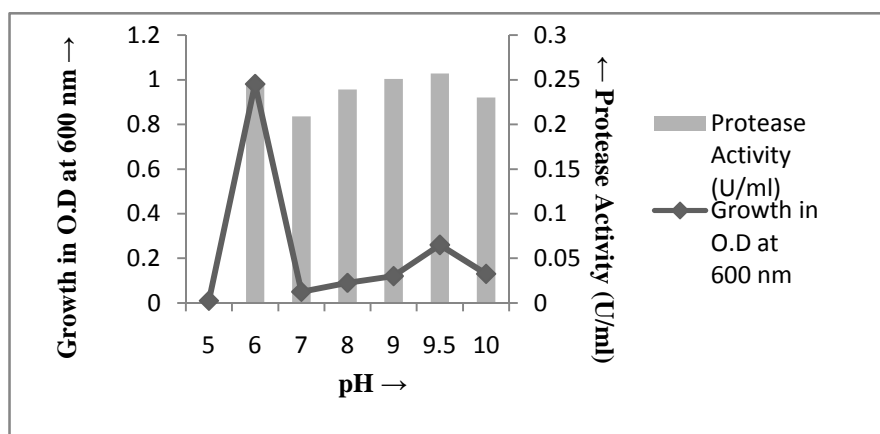


Fig.5: Comparison of bacterial growth and protease activity depending on medium Ph

Effect of temperature: The isolated strain showed highest biomass production at 30°C whereas the highest enzyme activity was found at 37°C. (Fig.6)

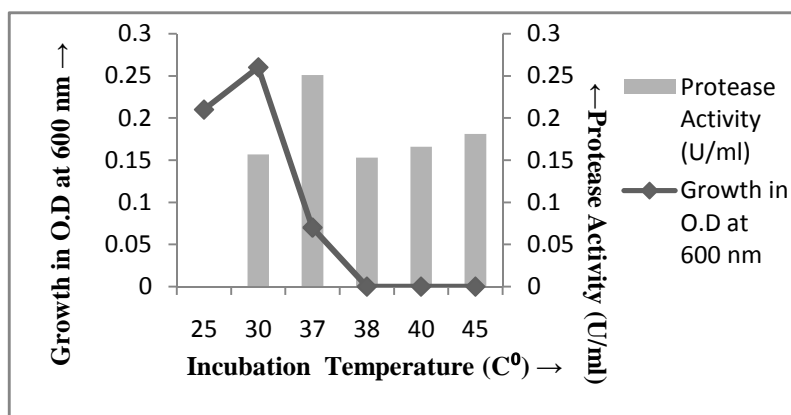


Fig.6: Comparison of bacterial growth and protease activity depending on incubation temperature.

Screening for biosurfactant activity: The isolated strain was tested for biosurfactant activity and showed positive results in different screening tests.

Drop Collapsing Method (DCM): In this method the best result was seen with kerosene oil. The results are presented in Table 1.

Table 1. Sensitivity of the oils in Drop Collapsing Method

Oil Samples	Results
Diesel	+++
Kerosene	++++
Castor oil	+
Paraffin oil	++

*(+) sign indicates the activity of biosurfactant produced in collapsing the oil drop surface.

Hemolytic activity (HA): The isolate was streaked on blood agar plate and incubated for 24-48 hrs at 37°C and it showed haemolysis, indicated by formation of clear zone. (Fig.7)



Fig.7: (a) Haemolysis in 24hrs and (b) Haemolysis in 48 hrs which is visible by clear region around the growth of the colonies.

Oil Spreading Technique (OST): In oil spread method the highest surfactant activity was shown kerosene which again indicates that produced biosurfactant has better activity against crude oil. The oil dispersed zone is measured in mm (Table 2).

Table 2. Oil dispersed zone in oil spread method

Name of oil used	Diameter(in mm)
Castor oil	16
Petrol	41
Diesel	46
Kerosene	50

Emulsification Stability (E24) Test:

Emulsifying activities (E24) determine productivity of bioemulsifier [25]. The present study has shown the highest emulsification activity (E24) in petrol of 64% which is more than E24 of 1% of SLS solution after 24 hrs (Table 3).

Table 3. Emulsification Activity in percentage

Name of Oils used	Height of Emulsified layer(mm)	Height of total liquid column (mm)	Emulsification Activity (E24) (%)
Paraffin	10	24	41.7
Petrol	16	25	64.0
Diesel	15	24	62.5
Kerosene	14	23	60.8
Castor	11	21	52.0

Blue Agar Plate Method: Formation of dark blue halo around the growth region indicates that the biosurfactant produced in rhamnolipids in nature. (Fig.8)



Fig.8: Shows rhamnolipid biosurfactant production which was indicated by dark blue halo around the growth region.

Optimization of culture conditions (for biosurfactant):

Effect of carbon source and concentration: Results showed that lactose was the chief carbon source of surfactant production and 3.0 mg/100 ml was the optimum lactose concentration for optimum surfactant production. (Fig.9)

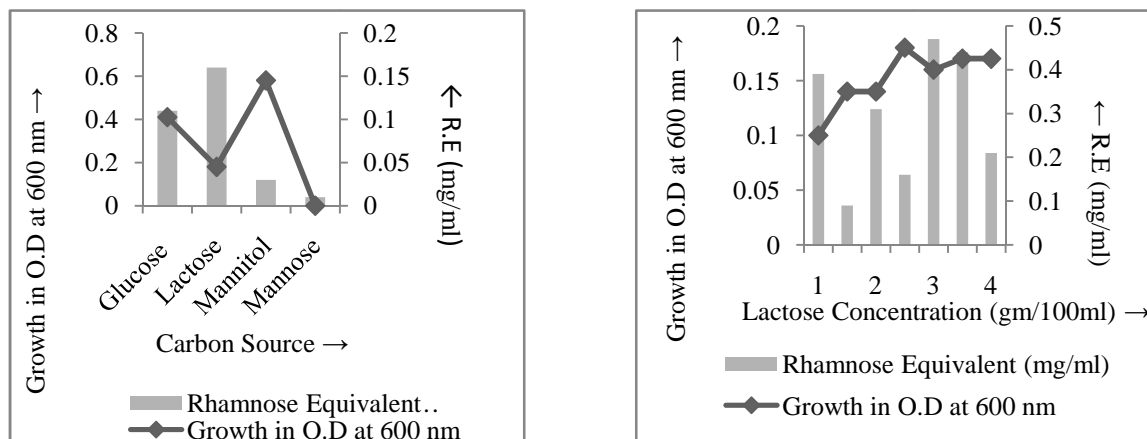


Fig.9: Comparison of bacterial growth and Rhamnose Equivalent (mg/ml) depending on Carbon source and its concentration

Effect of protein source and concentration: Peptone was the most effective protein source for optimum biosurfactant.

The other sources used and the optimum concentration was 0.138 mg/100 ml. (Fig.10)

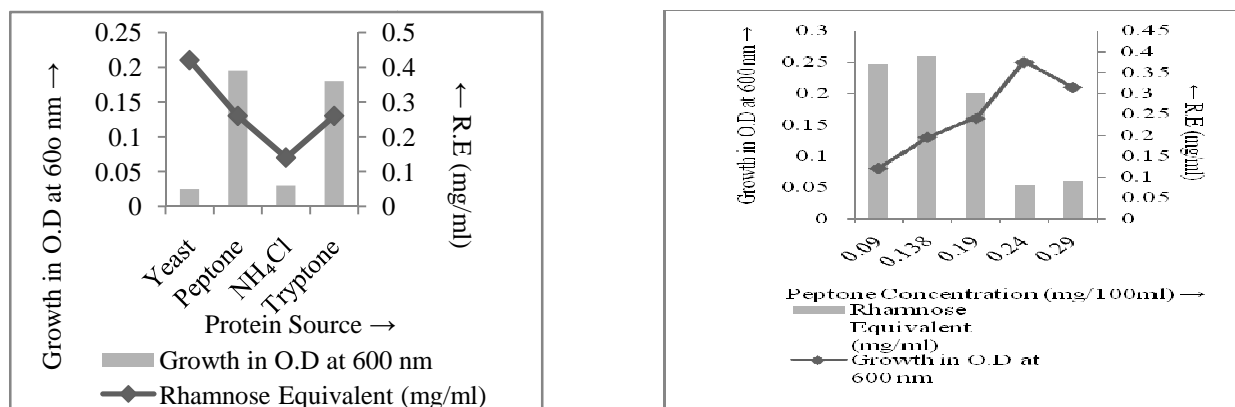


Fig.10: Comparison of bacterial growth and Rhamnose Equivalent (mg/ml) depending on protein source and its concentration

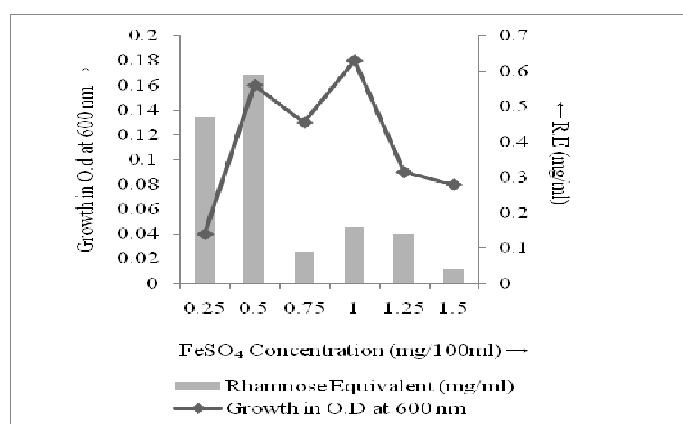


Fig.11: Comparison of bacterial growth and Rhamnose Equivalent (mg/ml) depending on FeSO₄ concentration

Effect of FeSO_4 concentration: From the result it was clear that for optimum surfactant production FeSO_4 concentration is also vital because after a certain concentration both surfactant production and growth decreases. The highest FeSO_4 concentration was 0.5 mg/100 ml for optimum surfactant production. (Fig.11)

Effect of medium pH: Medium pH was tested from 5.0 to 11.0 and optimum pH was found as 10. (Fig.12)

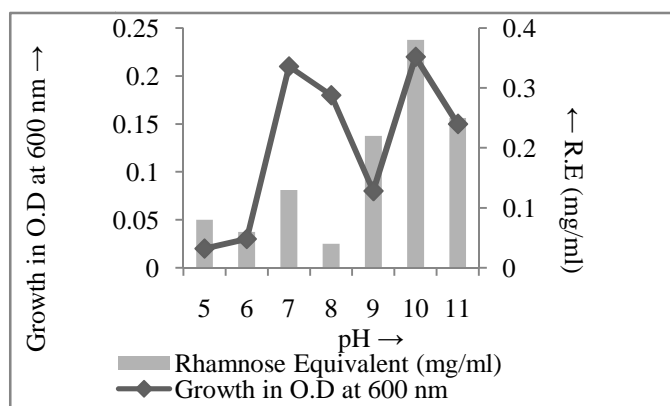


Fig.12: Comparison of bacterial growth and Rhamnose Equivalent (mg/ml) depending on pH of medium

Effect of incubation temperature: The experiment resulted as 40°C to be the optimum temperature for maximum surfactant production. (Fig.13)

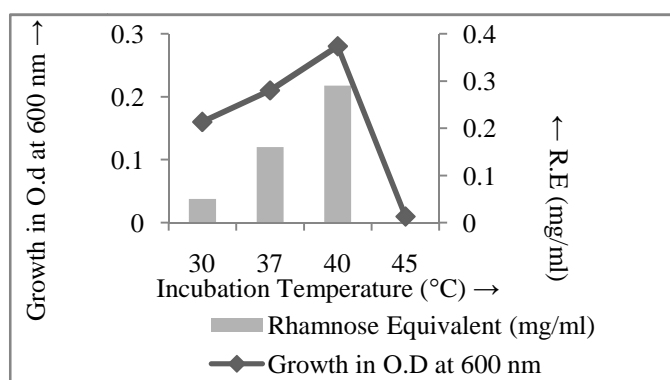


Fig.13: Comparison of bacterial growth and Rhamnose Equivalent (mg/ml) depending on incubation temperature

Effect of incubation period: Incubation period of assay broth was tested from 24 hrs to 96 hrs and the optimum period for maximum biosurfactant production was resulted as 24 hrs incubation. (Fig.14)

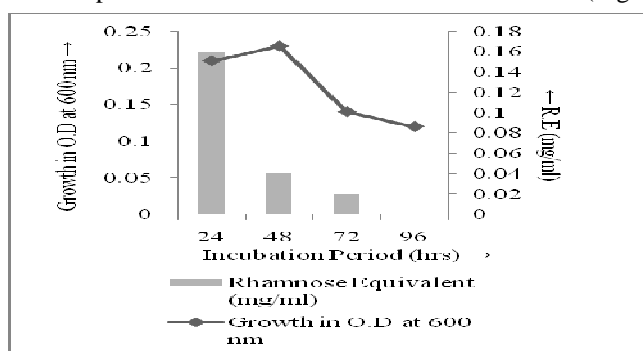


Fig.14: Comparison of bacterial growth and Rhamnose Equivalent (mg/ml) depending on incubation period

DISCUSSION

In the present study, the isolated bacterium *Pseudomonas aeruginosa* was examined to optimize its protease and biosurfactant activity. The strain is of great industrial importance because of its dual ability to produce protease

enzyme and biosurfactant. Bacterial protease fulfills the needs of cheese ripening and its flavor [26]. The isolates uses casein as protein source for optimum protease production, so this can be highly used industrially for cheese production.

The isolate produces alkaline protease (pH 9.5) which is preferable for enzyme-assisted dehairing process of leather. Traditionally this process is carried out with saturated solution of lime and sodium sulphide which are expensive and toxic too but use of bacterial protease is safe and economic.

Detergent industries use different enzymes, acting at alkaline pH, as additives. The use of different enzymes as detergent additives arises from the fact that proteases can hydrolyze proteinaceous stains; amylases are effective against starch and other carbohydrate stains while lipases are effective against oily and fatty stains. For an enzyme to be used as a detergent additive: it should have an alkaline pH and it should also be compatible with detergent [27]. The strain also produces biosurfactant (rhamnolipid, pH 10). Rhamnolipid is a detergent-like glycolipid with excellent foaming properties, which is produced by *P. aeruginosa* [28]. So, this agent fulfills both of the above criteria and most suitable for detergent industries.

The protease obtained from this isolate can surely be used to produce useful proteinaceous biomass and as well as for bioremediation.

On the other hand the strain also produces surface active agent which in DCM, OST and E24 tests showed high activity against crude oil. So, it can be successfully used to remove accidental oil spills from water surface without disturbing marine ecology. Moreover as the strain has been isolated from municipal solid waste having the capability to produce industrially effective protease and biosurfactant; this is certainly a process of waste management and bioremediation.

Acknowledgement

The authors are pleased to acknowledge the Xcelris Labs Ltd. (Sydney House, Ahmedabad, India) for identification of microbial culture using 16S rDNA based molecular technique and also to the Jadavpur University to carry out this research work successfully.

CONCLUSION

In the present study, the isolated bacterium *Pseudomonas aeruginosa* was examined to optimize its protease and biosurfactant activity. This information enables us to assess the biosurfactant activity for further exploitation in specific applications like as antitumor activity in the near future.

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