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CONCURRENT PRODUCTION OF BIOSURFACTANT AND ENZYME PROTEASE BY BACTERIA AND ESTIMATION OF APPLE POMACE WASTE FOR LOW-COST PRODUCTION

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ABSTRACT

The present study is an attempt to optimize production of biosurfactant and protease simultaneously from bacterial isolates of the oil-contaminated region of Karachi coastal area. Bacterially produced biosurfactant and proteases well known for many applications in various industries and due to their environmentally friendly nature. Their production cost, however, remains high because of the high cost of culture media and low yield that is why only a few microbial sources recognized as commercial producers for biosurfactant and protease.

For the present study, bacterial isolates selected for rhamnolipid production followed by screening for protease activity. Total 24 isolates selected for biosurfactant production through oil spreading, hemolytic activity, CTAB agar plate, drop collapse test, BATH assay, and emulsification activity (E24). Skim milk agar plate used for screening of protease producing isolates by producing clear zone. As a cheap source for the production of biosurfactants, the apple pomace successfully used in culture media.

This study accomplishes that these isolates have the ability to produce commercially important biosurfactants and proteases respectively. It also suggested that apple pomace is a cost-effective substrate for the production of commercially important biomolecules.

Keywords: Biosurfactants, proteases, adherence, skim milk agar

INTRODUCTION

Surfactants are chemicals, which help to reduce surface tension, interfacial tension and solubilize hydrocarbons. Based on production surfactants classified into two categories; chemically derived surfactants and biologically produced surfactants known as biosurfactants. Microorganisms work as chemical factories in producing commercially significant biosurfactants which are preferred over their synthetic counterparts for having lower toxicity, biodegradability and stability at high temperature, pH and salinity (Das *et al.*, 2008; Pandy, 2012). These bio-molecules also have pharmaceutical properties as well as antiviral, antifungal and antibacterial properties (Singh and Cameotra, 2004).

Proteases are the distinct class of enzymes, which have important applications in both physiological and commercial fields. Catalytic function of proteases is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. Proteases are widely used in leather processing, detergent industry, food industries, bioremediation process, pharmaceutical, textile industry, waste processing companies, and in the film industry (Rao *et al.*, 1998). Microbes serve as a preferred source of production of these enzymes in limited space because of their rapid growth and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for various applications (Kocher and Mishra, 2009). Enzyme producing bacteria are widely distributed in soil and water, and certain strains tolerate extreme environmental conditions including highly alkaline conditions. One of the most important characteristics that determine the industrial suitability of proteases is their requirement of high pH for optimum enzyme activity. Screening of proteases producing bacterial species from different ecological environments can result in isolation of new proteases with unique characteristics for the various industrial application (Singh *et al.*, 1999).

Due to high production cost and low yield, the commercial production of biosurfactants and proteases is limited. (Nitschke and Pastore, 2002; Pornsunthorntawee *et al.*, 2010). Alternative low-cost substrates like agro-industrial wastes, hydrophobic wastes, used frying oil, sludge from petroleum refineries and peels of different fruits or vegetables utilized for reducing the production cost of biosurfactants (Cameotra and Makkar, 1998).

This study mainly focused on the screening of bacterial isolates capable of producing both biosurfactants and proteases and assessment of apple pomace as a cheap source for reducing the production cost of these biomolecules for the future industrial application.

MATERIALS AND METHODS

Bacterial Isolates

Previously isolated and preserved bacterial isolates were utilized in the present study (Shoeb *et al.*, 2012). For enrichment Luria Bertani (LB) broth was used (Bertani, 1951). Cultures grown at 37°C and stored at 4°C. The isolates coded as DGEF11-DGEF34.

Identification through GSP agar plate method

For the detection of *Pseudomonas* and *Aeromonas* from the samples Glutamate Starch Phenol Red (GSP) agar (Oxoid) used for preliminary screening (Stanier *et al.*, 1966) . The purified isolates streaked on GSP agar plates and incubated at 37°C for 24–48 hours (Martínez-Martínez *et al.*, 1998). Result analyzed by a change in color from red-violet to yellow.

Screening Methods for Bio-surfactant Production

Isolates were grown aerobically for the screening of biosurfactant production through oil spreading method, hemolytic activity, CTAB agar plate, drop collapse test, BATH assay, emulsification activity (E24).

Oil spreading method

Oil spreading technique is a primary screening test of biosurfactant. Oil spreading was performed according to the method described previously by (Youssef *et al.*, 2004). The occurrence of the clear zone on the oil surface was an indication of biosurfactant production. The diameter of a clear zone measured and compared to 10µL of distilled water as negative control.

Hemolytic activity

Hemolytic assay performed on blood agar plates. O/N culture was spot-streaked on blood agar plates and incubated for 48 h at 37°C. The plates visually inspected for the zone of clearance (hemolysis) around the colony which was used as an indicator of biosurfactant production (Mulligan *et al.*, 1984).

CTAB Agar Plate

Blue agar plates containing cetyltrimethylammonium bromide (CTAB) and methylene blue used to detect extracellular glycolipid production (Siegmund and Wagner, 1991). Biosurfactant production observed by the formation of dark blue halos around the colonies.

Drop-collapse test

Screening of biosurfactant production performed using the qualitative drop-collapse test described by Jain *et al.* (1991) as modified by Bodour and Maier (1998). A result considered positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by deionized water (negative control).

Bacterial adhesion to hydrocarbons (BATH) Assay

BATH assays were performed as previously described by (Rosenberg *et al.*, 1980). Hydrophobicity expressed as the percentage of cell adherence to hydrocarbon calculated using following formula:

1-(OD of the aqueous phase/OD of initial cell suspension) ×100

Emulsification activity (E24)

Emulsification activity performed using cell-free supernatant with xylene as hydrocarbon (Freitas *et al.*, 2009). The emulsification activity was determined using the following formula:

E24 = (Height of emulsion layer/ Height of liquid column) ×100

Screening for Protease activity by bacterial isolates

Proteolytic activity of the bacterial cultures screened on skimmed milk agar plates containing skimmed milk powder 1.0%, peptone 0.5%, and sodium chloride 5% and agar 2.5%. The pH of the medium adjusted to 9.0 with 1N HCl/1N NaOH, before sterilization at 121°C for 15 minutes. The plates then incubated at 37°C for 48 hrs. The formation of the clear zone around the colonies confirmed the production of alkaline protease (Amoozegar *et al.*, 2008). Hydrolysis expressed as the diameter of clear zone in mm.

Effect of Sodium chloride on protease activity

The isolates with positive protease activity further tested with varying concentration of NaCl on skimmed milk agar plate. The protease producing positive bacterial isolates streaked on skimmed milk agar plates containing 3%, 5% and 10% NaCl separately, incubated at 37°C for 48 hrs. Hydrolysis expressed as the diameter of clear zone in mm. The bacterial isolates with prominent zones of clearance considered as positive.

Use of Apple pomace as a cheap source for biosurfactant production

Preparation of substrate

Apple (*Malus pumilla* Mill.) fruit pomace used as a cheap nutritional source for biosurfactant production in this study. Apple pomace collected from the canteen at the University of Karachi. Firstly, apple pomace washed with water and then air-dried. After drying apple pomace crushed into powder and autoclave for 15 min. Stored at room temperature for further use.

Production media and cultivation conditions

A mineral salt medium (MSM) containing (g/L): KH_2PO_4, 1.4; Na_2HPO_4, 2.2; $(NH_4)_2SO_4$, 3; $MgSO_4.7H_2O$, 0.6; NaCl, 0.05; yeast extract, 1; $FeSO_4.7H_2O$, 0.01 and $CaCl_2.7H_2O$, 0.02; was used. The pH of the medium adjusted to 7.0.The mineral medium supplemented with apple pomace 2% (w/v) as the sole carbon source for biosurfactant production. All cultivations carried out in 250 mL flasks containing 50 ml of MSM medium incubated at 37 °C, agitation rate 150 rpm for 5 days. Absorbance at 600nm was taken on every 24 hrs and the cell-free supernatant subjected to emulsification activity (Ilori *et al.*, 2005).

RESULTS AND DISCUSSION

The present study aimed to focus on the investigation of industrially important biomolecules produced by microorganisms. Biosurfactants and enzymes have great importance in our daily life and beneficent to the ecosystem (Pandy, 2012). Biosurfactant, protease well known for their industrial significance too, and it is very desirable to find bacterial isolates capable of simultaneous production of biosurfactant and protease. In this study, we have successfully isolated twenty-four bacterial isolates, which previously purified from samples of Arabian Sea coast of Karachi (Shoeb et al., 2015). Species identification on GSP agar plates showed that among the 24 isolates, 10 (41%) isolates belong to the genus Pseudomonas (Thavasi and Jayalakshmi, 2003). The existence of biosurfactant producing Pseudomonas species in hydrocarbon polluted environment is reported by many researchers (Das and Mukherjee, 2005). A number of methods are reported for the screening of biosurfactant producing bacteria (Kiran *et al.*, 2009); (Walter *et al.*, 2010). We used six methods, which are, oil spreading method, hemolytic activity, CTAB agar plate, drop collapse, BATH assay and emulsification activity (E24), to screen the biosurfactant producing isolates.

Out of 24 bacterial isolates, 16 (66%) isolates significantly displaced oil layer and started to spread in the water, showing clear zone on oil plate. The maximum size of a zone formed by isolates DGEF11 and DGEF31 (35mm and 33mm) respectively as shown in Table 1 and in Fig. **1**. Oil spreading results were in support of drop collapse assay results. Isolates, which were positive for oil spreading assay also showed positive results with drop collapse test (Table 2 and Fig. **2**). These results confirmed the presence of biosurfactant in cell-free supernatant. It is the most effective tools to prove the biosurfactant production in many bacterial isolates. Youssef *et al.* (2004) reported similar findings with oil spreading and drop collapse assay. It reported that clear area formed due to the displacement of oil reflects the activity of biosurfactant. Larger the displacement area signifying a high biosurfactant activity (Sidkey and Al Hadry, 2014).

Similarly, 95% isolates showed clear zone around the streaks of the colony in blood agar plates, confirming hemolytic activity as shown in Table 3 and Fig. **3**. Lysis of red blood cells suggested as a simple and easy method to test for biosurfactant activity (Yonebayashi *et al.*, 2000) and it is widely used to screen biosurfactant production (Shoeb *et al.*, 2015).

The CTAB is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants (Saravanan and Vijayakumar, 2012). All twenty-four isolates subjected to CTAB agar plate method and results revealed that 95% isolates produced the dark blue halos around the colony and considered as positive. Isolates possess highest biosurfactant activity confirmed the presence of anionic biosurfactant as shown in table and figure. The maximum size of zone formation 26mm, 25mm observed in DGEF32 and DGEF31 respectively (Table 4 and Fig. **4**). Anitha *et al.* (2015) used CTAB assay for screening of newly isolated bacterial strain.

Table 1. Result for oil-spreading assay, indicated oil displacement produced by isolates.

Code #	Oil displacement (mm)
DGEF11	35
DGEF12	24
DGEF13	25
DGEF14	25
DGEF15	19
DGEF16	10
DGEF17	13
DGEF18	10
DGEF19	30
DGEF20	20
DGEF21	15
DGEF22	5
DGEF23	3
DGEF24	5
DGEF25	5
DGEF26	15
DGEF27	10
DGEF28	12
DGEF29	12
DGEF30	2
DGEF31	33
DGEF32	20
DGEF33	5
DGEF34	5

Table 2. Drop collapse used for biosurfactant activity.

Code #	Drop Collapse
DGEF11	+
DGEF12	+
DGEF13	+
DGEF14	+
DGEF15	+
DGEF16	+
DGEF17	+
DGEF18	+
DGEF19	+
DGEF20	+
DGEF21	+
DGEF22	+
DGEF23	+
DGEF24	+
DGEF25	+
DGEF26	+
DGEF27	+
DGEF28	+
DGEF29	+
DGEF30	+
DGEF31	+
DGEF32	+
DGEF33	+
DGEF34	+

Fig. 1. oil spreading assay showing highly active biosurfactant producers, the changes seen in the oil present in the systems, compared to the control (water) without any change.

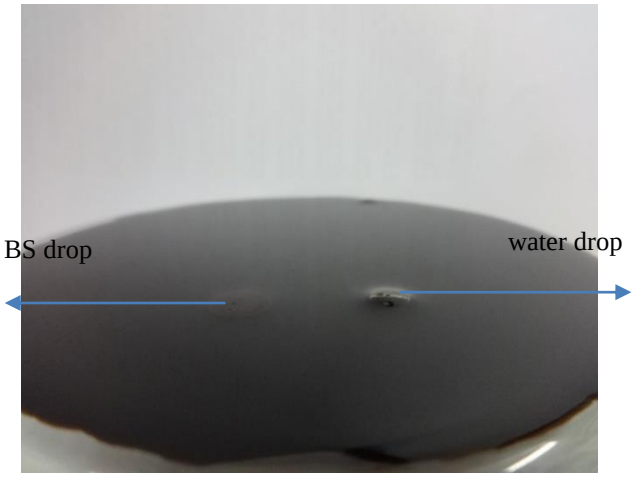

Fig. 2. for drop collapse assay, drop containing biosurfactant may collapse on oil layer as compare to water.

Table 3. Results for hemolytic activity.

Code #	Hemolytic Activity
DGEF11	β
DGEF12	α
DGEF13	α
DGEF14	β
DGEF15	β
DGEF16	β
DGEF17	α
DGEF18	α
DGEF19	α
DGEF20	α
DGEF21	α
DGEF22	α
DGEF23	β
DGEF24	β
DGEF25	β
DGEF26	β
DGEF27	β
DGEF28	β
DGEF29	β
DGEF30	Y
DGEF31	α
DGEF32	α
DGEF33	α
DGEF34	α

* α' hemolysis indicates complete lysis cells,
β' hemolysis indicates partial lysis of red cells,
Y' no hemolysis.

Table 4. Results for CTAB assay indicated zone formation (mm).

Code #	CTAB test Zone size (mm)
DGEF11	16
DGEF12	15
DGEF13	16
DGEF14	14
DGEF15	21
DGEF16	15
DGEF17	20
DGEF18	19
DGEF19	20
DGEF20	22
DGEF21	16
DGEF22	15
DGEF23	21
DGEF24	20
DGEF25	17
DGEF26	19
DGEF27	20
DGEF28	20
DGEF29	20
DGEF30	19
DGEF31	25
DGEF32	26
DGEF33	22
DGEF34	18

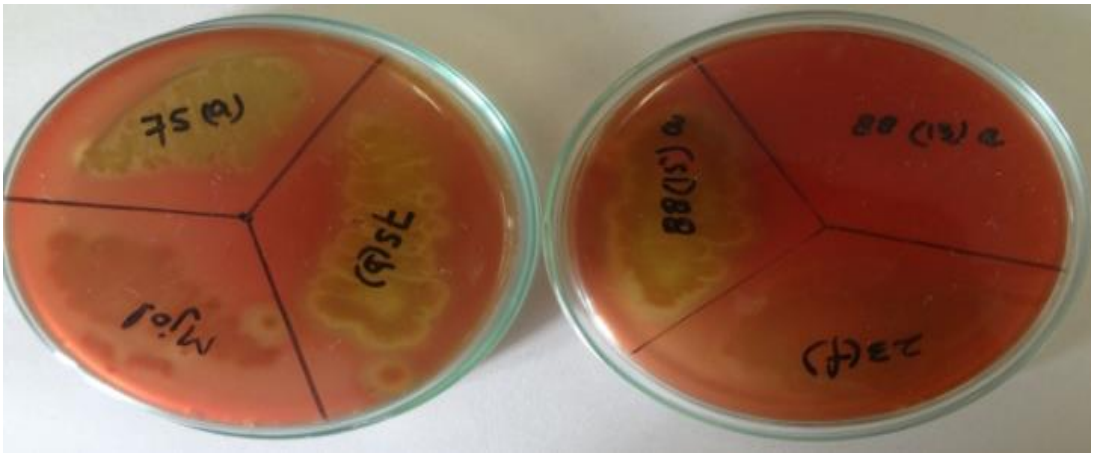

Fig. 3. Hemolytic activity of bacterial isolates showed lysis of red blood cells.

Table 5. Bath assay of isolates against xylene as hydrocarbon.

Code #	Bath Assay (%)
DGEF11	33.5
DGEF12	29.3
DGEF13	19.2
DGEF14	38.9
DGEF15	34.7
DGEF16	30.6
DGEF17	37
DGEF18	16.5
DGEF19	6.9
DGEF20	31.4
DGEF21	38.6
DGEF22	35.4
DGEF23	39.6
DGEF24	21.9
DGEF25	44.6
DGEF26	29.4
DGEF27	16.7
DGEF28	37.9
DGEF29	30.3
DGEF30	17.8
DGEF31	25%
DGEF32	27.1
DGEF33	16.1
DGEF34	26.5

Table 6. Emulsification index (E24) against generator oil and motor oil.

Code #	Generator oil (%)	Motor oil (%)
DGEF11	33.3	33.3
DGEF12	33.3	40
DGEF13	35.7	31
DGEF14	33.3	**43.7**
DGEF15	35.7	26.6
DGEF16	31.2	41.1
DGEF17	33	27
DGEF18	31.2	26.6
DGEF19	33.3	35.2
DGEF20	30.4	26.6
DGEF21	40	31
DGEF22	26.3	**43.7**
DGEF23	**50**	41.1
DGEF24	35.2	26.6
DGEF25	41.1	33.3
DGEF26	35.7	40
DGEF27	28.5	31
DGEF28	29.4	33.3
DGEF29	29.4	33.3
DGEF30	**46.6**	26.6
DGEF31	33	**53**
DGEF32	35.7	**43.7**
DGEF33	28.5	40
DGEF34	26.6	26.6

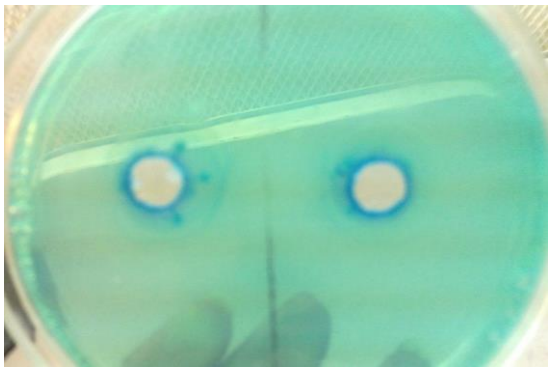

Fig. 4. In CTAB assay, dark blue halo was formed around bacterial growth indicated the biosurfactant activity.

Table 7. Screening of selected protease producing bacteria on skim milk agar plates.

Codes #	Zone size after 24hr (mm)	Zone size after 48hr (mm)
DGEF11	30	45
DGEF12	13	16
DGEF13	12	12
DGEF14	18	29
DGEF15	14	32
DGEF16	22	22
DGEF17	30	30
DGEF18	10	10
DGEF19	20	30
DGEF20	22	22
DGEF21	22	22
DGEF22	11	11
DGEF23	29	29
DGEF24	20	29
DGEF25	32	32
DGEF26	25	25
DGEF27	15	15
DGEF28	18	18
DGEF29	31	31

Table 8. To check the effect of sodium chloride (NaCl) on positive protease producing isolates.

Code #	NaCl Concentration
DGEF11	10%
DGEF12	10%
DGEF13	10%
DGEF14	10%
DGEF15	10%
DGEF16	3%
DGEF17	5%
DGEF18	5%
DGEF19	5%
DGEF20	5%
DGEF21	5%
DGEF22	5%
DGEF23	10%
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DGEF25	5%
DGEF26	3%
DGEF27	5%
DGEF28	5%
DGEF29	5%

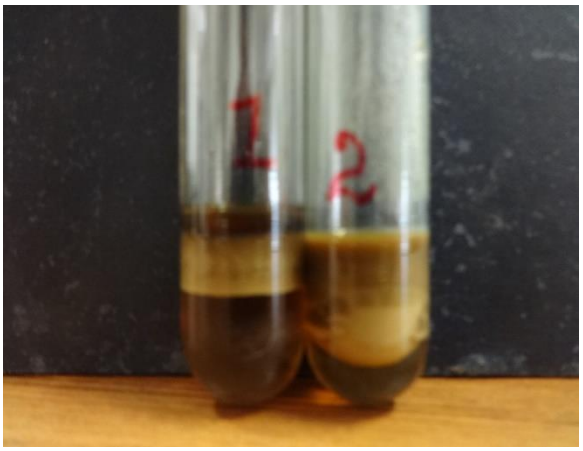

Fig. 5. Image of the emulsification assay showed emulsion formed against generator oil.

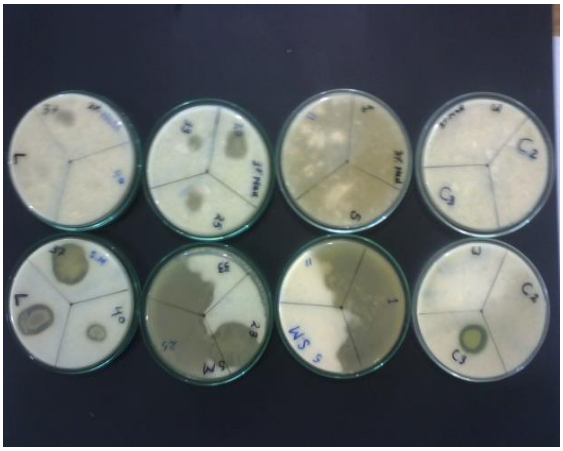

Fig. 6. Protease production by producing clear zone on skim milk agar plates.

Bacterial adhesion to hydrocarbons (BATH assay) performed to estimate the affinity of the cell surface to hydrocarbon. It is a photometric based method, used to measures the degree of adhesion to hydrocarbon. Interaction with hydrophobic compounds considers an indirect method to screen biosurfactant producer. For this purpose, xylene used as the hydrophobic compound. Result for BATH assay indicated that all twenty-four isolates were positive and showed affinity of the bacterial cells with xylene. Cell attachment for positive isolates with xylene was in the range of 16.5-44.6%. Maximum cell attachment with xylene observed in isolate DGEF25 (44.6%) followed by DGEF14, DGEF21, DGEF23 (38.9, 38.9 and 39.6%) respectively as shown in the Table 5. Bacterial strains with high cell hydrophobicity are reported as potential biosurfactant producers (Volchenko *et al.*, 2007). According to (Zhang and Miller, 1994) strains of Pseudomonas genus showed highest cell adherence with crude oil as compared to other bacterial isolates. Many reports mention BATH assay as principle method for screening of biosurfactant producers (Volchenko *et al.*, 2007).

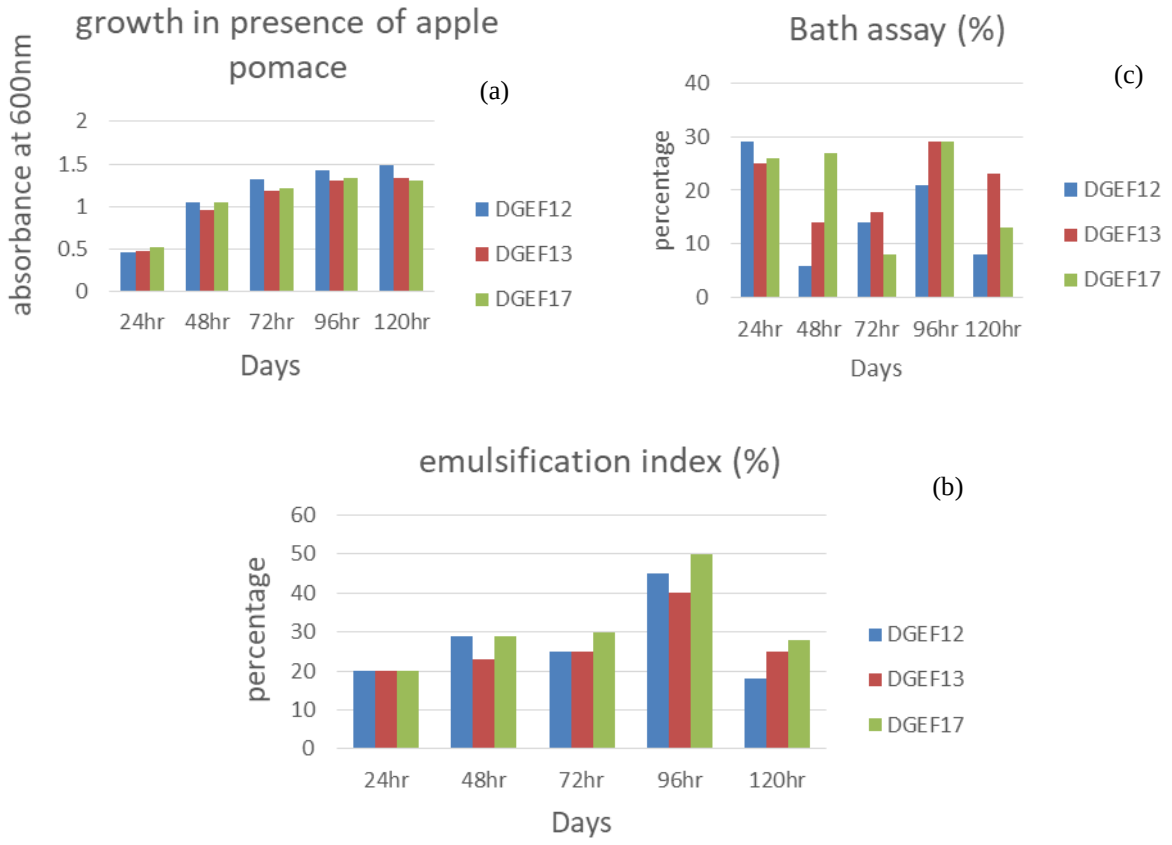

Fig. 7(a-c) Production of Biosurfactant in presence of apple pomace as nutritional source. (a)Showing growth curves at OD600 indicating growth with apple pomace. (b) Emulsification indexes values of produced biosurfactants against xylene.(c) bath assay result showed affinity of the cell with xylene with respect of time.

Emulsification activity (E24) is another method used to determine the potential and stability of biosurfactant (Ilori *et al.*, 2005). An emulsion formed when one liquid phase dispersed as microscopic droplets in another liquid phase. Analysis of emulsification activity indicated that isolate DGEF23 and DGEF31 exhibited highest emulsification capacity of 50% and 46.6% in against generator oil. Isolates, which were positive with generator oil, showed emulsification in range of 26 -50 %. While the isolates that positive with motor oil were in the range of 26-43% of E24. The maximum emulsion formed by isolate DGEF31 (53%) with motor oil correspondingly (Table 6 and Fig. **5**). Similarly, (Khalid, 2011) found an emulsion of 51% from the bacterial strain of Bacillus subtilis DSM 15029. According (Willumsen and Karlson, 1996) an emulsification index of 50.0% represents some good emulsifier properties of a biosurfactant. Although the stability of emulsion formed by bacterial isolates is irrespective of bio emulsifier produced (Cameotra and Makkar, 1998). Emulsification index (E24) is the speedy and consistent measure of produced biosurfactant (Asfora Sarubbo *et al.*, 2006).

The proteolytic activities of all the isolates were assayed using skim milk agar plate method. Proteolytic bacteria hydrolyze casein and form soluble nitrogenous compounds exhibited as a clear zone around colonies. Researchers (Vermelho *et al.*, 1996) suggested that the hydrolysis zone produced on the casein agar could be related to the amount of protease produced. Similarly, Gupta (Gupta and Gupta, 2005) performed isolation of bacterial isolates from environmental samples and recommended skim milk agar for the screening of protease producing organisms. Out of 24 isolates streaked on skim milk agar plates, 19 (79%) isolates produced clear zone on skim milk agar plates after 48 hours of incubation. Among all tested isolates, DGEF11 showed highest protease activity by producing the extensive clear zone of 45mm after 48 hours (Table 7 and Fig. **6**).

Bacterial Isolates with positive Protease activity further treated with different sodium chloride (NaCl) concentration (3%, 5% and 10% w/v). All isolates were able to grow on salt containing skim milk agar plates. Isolates considered positive by producing clear zone on skim milk agar plates by tolerating concentration of NaCl

from 3% to 10% after 48 hours of incubation (Table 8). According to (Sanchez-Porro *et al.*, 2003) proteases was more active and stable in a wide range of NaCl concentration (Souza *et al*., 2012).

The use of waste material as carbon sources to produce biosurfactants is an interesting and low-cost alternative (Abouseoud *et al.,* 2008). In present study for biosurfactant production, apple pomace used as a cheap nutritional source in the cultivation media. Biosurfactant may produce during stationary growth phase as a typical secondary metabolite. It also depends upon the type of carbon substrate select for biosurfactant production (Davis *et al.*, 1999). Results suggested that bacterial growth in apple pomace medium gradually increased and best absorbance obtained after the 5th day of incubation by isolate DGEF12 as shown in Fig. **7a**. Similar findings were obtained by Rocha (Rocha *et al.,* 2006) using natural cashew apple juice in the mineral complex medium for biosurfactant production. Sobrinho (1999) produced biosurfactant by utilizing 4% corn steep liquor and refinery waste as a substrate.

In our study, after completion of incubation, the biosurfactant activity measured through emulsion formation of cell-free supernatant against xylene showed maximum activity i.e. 50% after 96 hours (Fig.7b).

Results for cell attachment to hydrophobic compound indicated that the attachment of a cell to xylene was in the range of 13-29% (Fig. 7c). The biosurfactant production and bath assay dependent on the growth of culture in the fermentation medium.

Results of the present study indicated positive prospects for use of apple pomace as a sole carbon source for biosurfactant production. Approximately 10–30% accounts the total production cost for biotechnological processes. The use of agro-industrial waste not only reduce cost but also help to clean the environment.

Conclusion

Biomolecules produced by microorganism have many pharmaceutical, food and industrial applications. In this study, we have screened the twenty-four isolates for biosurfactant production, nineteen of which showed positive protease production. This concurrent production of protease from biosurfactant producing isolates and their activity on different salt concentration is an interesting application for biotechnological processes. For an economical point of view, the use of apple pomace as a promising substrate for biosurfactant production demonstrated. Apple pomace as an abundant, accessible and agro-industrial waste seems to have the potential for a low-cost solution for commercial production. Further studies are in progress to identify the characteristic of these biomolecules and consequently determine the potential of their different industrial applications.

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