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Co-production of Pectinase and Biosurfactant by the Newly Isolated Strain *Bacillus subtilis* BKDS1

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Authors' contributions

This work was carried out in collaboration between all authors. Author DS conceived and designed the study. Authors BK and SKT carried out the experiments and performed the analysis of data. Author BK wrote the first draft of the manuscript and managed the literature searches. Author DS edited and proofread the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To isolate pectinase producing bacterial strains from various samples and to investigate the co-production of pectinase and biosurfactant by this isolated strain.
Study Design: Isolation, screening, selection and identification of pectinolytic bacteria. Production of pectinase and analysis of exo-pectinase types. Partial purification by ammonium sulphate precipitation and dialysis. The study also analyses the capability of the selected strain for biosurfactant production.
Place and Duration of Study: Department of Life Sciences, University of Calicut, Kerala - 673635, India, between July 2013 and November 2014.
Methodology: The pectinolytic bacteria was isolated and screened by iodine plate assay method. The enzyme production was confirmed by 3,5-dinitrosalicylic acid (DNS) method. The potent enzyme producing strain was biochemically characterised and identified by 16S rRNA gene sequence analysis. Thiobarbituric acid (TBA) and DNS assay were performed to test the types of exo-pectinase produced. Partial purification of the enzyme was done by ammonium sulphate precipitation. Biosurfactant production by the strain was tested by methods like foam formation,

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drop collapse test, oil displacement test, microplate assay, hemolytic assay, penetration assay, emulsification activity and bacterial adhesion to hydrocarbon (BATH) assay.
Results: Thirty six pectinolytic bacterial strains were screened from the collected samples. Four isolates were selected on the basis of zone size (20 mm to 26 mm) in well plate screening method. In which, the isolate showed maximum enzyme production in DNS assay (0.707 U/mL) is the same which exhibited larger pectin depolymerization area (26 mm) in well diffusion assay. This strain was identified as *Bacillus subtilis* BKDS1 using 16S rDNA based molecular technique. The optimum incubation time was found to be 72 h for the maximum enzyme production (1.288 U/mL). Assay of exo-pectinase revealed that, the organism was able to produce polygalacturonase (PG), pectin lyase (PNL) and pectate lyase (PEL). In ammonium sulphate precipitation, the enzyme activity was found in 40 -100% salt saturation fractions. On further analysis, it was observed that the organism also produced biosurfactant along with pectinase in the same culture medium.
Conclusion: The present study indicates the possibility of an integrated process for obtaining pectinase enzymes and biosurfactants in the same culture media. So this could greatly increase the economic viability of the isolated strain.

Keywords: Pectinase; polygalacturonase; biosurfactant; Bacillus subtilis.

1. INTRODUCTION

There is an increasing interest for isolating new enzymes and new enzyme producing strains for their use in industrial conversion [1]. Among the different enzymes, pectinases constitute a unique group of enzymes which catalyze the degradation of pectic polymers present in the plant cell walls. The main chain of pectin is partially methyl esterified 1,4 –D-glacturonan. Demethylated pectin is known as pectic acid (pectate) or polygalacturonic acid. This enzyme splits polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages. Pectinases include; pectin esterases, which catalyse the hydrolysis of methylated carboxylic ester groups in pectin into pectic acid and methanol, Pectin lyases which cleave α (1, 4)-glycosidic linkages by transelimination resulting in galacturonide with a double bond between C-4 and C-5 at the non reducing end and Polygalacturonases which hydrolyze the α (1, 4) - glycosidic linkages in homogalacturonans [2,3].

Naturally pectinase are produced by diverse range of microorganisms comprising fungi, bacteria, yeast and actinomycetes. Pectinases from fungal sources produce best under acidic pH and low temperature conditions and can therefore not be used in various industrial bioprocesses which utilize neutral to alkaline pH with high temperatures exceeding 45°C. It has been shown that bacteria require high pH and temperature to produce pectinase [4,5]. Also, bacterial strains producing commercial enzymes are always preferred over fungal strains because of ease of fermentation process (for production) and implementation of strain improvement techniques or any modern technique to increase the yield of production [6]. Moreover, bacterial pectinases with novel properties have the added advantage that enzyme production is achieved in less time as compared to fungal sources [7]. So, novel bacterial strains have to be identified for better production of pectinase. Because of these stated characteristics of bacterial pectinase over fungal pectinase, this work also concentrated on screening of bacterial isolates for pectinolytic property.

Biosurfactants or surface-active agents are biologically produced compounds which are naturally produced by bacteria, fungi and yeast. These are important in different sectors of modern industry, such as food, pharmaceutical, cosmetics and petroleum industries [8]. The massive commercial and healthcare promises of biosurfactants and the current market demand for cost competitive and environment friendly alternatives to synthetic surfactants has attracted scientists all over the world towards isolation and screening of biosurfactant producing microorganisms.

These compounds are capable of decreasing surface and interfacial tensions, as well as to form and stabilize oil in water or water in oil emulsions [9]. The majority of known biosurfactants synthesized by microorganisms are grown in water immiscible hydrocarbons, but some have also been produced in such water-soluble substrates as glucose, glycerol and ethanol [10]. Due to biodegradative property of biosurfactants, they are ideally suited for environmental applications. A great variety of surface active compounds have been isolated from hundreds of microbial species including

glycolipids, phospholipids, lipopeptides, lipoproteins, glycoproteins, polymeric and particulate structures etc. [11,12]. Among microorganisms, *Pseudomonas aeruginosa* for rhamnolipids, *Bacillus subtilis* for surfactin and *Candida* spp. for sophorolipids and mannosylerythritol lipids are mostly studied [8,13].

Researchers have attentive on microorganisms, which are capable of producing either of pectinase or biosurfactant, whereas exploration of a microorganism which is able to achieve co-production of pectinase and biosurfactant will be more profitable in terms of industrial demands. If co-production of these can be simultaneously achieved in a medium, energy and time consumption as well as medium cost will be reduced. Literatures are accessible concerning simultaneous production of various enzymes with biosurfactants [14-16]. The aim of the present study was the isolation and screening of potent pectinolytic enzyme producing bacterial strains from various samples and explores its efficiency for producing exo-pectinase types. Apart from this, the study also analyses the capability of the selected strain for biosurfactant production.

2. MATERIALS AND METHODS

2.1 Isolation of Microorganisms

Samples including soil (from dump yards of market vegetables and fruits) and rotten fruits and vegetables were collected and one gram was pooled and homogenized in sterile distilled water and 10 fold serial dilutions were prepared. Aliquots (1 mL) from each dilution were inoculated by spread plate method on yeast extract pectin (YEP) agar medium with pH 7 containing (g/l) Citrus pectin (sigma); 2.5, yeast extract;10. The samples were incubated at room temperature (30ºC) for 24 h. Pure cultures were subcultured onto slant media and maintained for identification and further studies.

2.2 Plate Assay of Depolymerised Pectin

2.2.1 Iodine assay

For iodine assay, the pure cultures were inoculated on YEP agar plates and incubated for 24 h at 30ºC. After incubation, the plates were overlaid with iodide solution (1.0 g iodine, 5.0 g potassium iodide and 330 mL H_2O) and kept undisturbed for 10 min. The colonies were selected as per the size of clear zone formation [17].

2.2.2 Agar well diffusion assay

The pectinolytic activity was also assayed by well diffusion method. The YEP agar plates were prepared and 10 mm diameter wells were cut aseptically with the help of cork-borer. The wells were filled with 100 µl of culture filtrate and incubated at 30ºC overnight. The substrate utilized zone around the colony was observed using iodine solution [17]. The pectin depolymerisation zone around the well (containing culture filtrate) was also confirmed by adding 3.3% cetyltrimethylammonium bromide (CTAB) [18].

2.3 Colorimetric Assay of Pectinase Activity

Overnight-grown cultures of the selected isolates were inoculated in to 20 mL YEP broth in 100 mL conical flasks. The flasks were incubated at 30°C and 150 rpm for 24 h. The pectinase activity was measured in the culture supernatant using a method adapted from Miller, 1959 [19]. Thus, 1 mL of the cell free supernatant was mixed with an equal volume of 1% (w/v) citrus pectin in 0.2M Tris-HCl buffer (pH 8) as the substrate. The mixture was incubated at 40°C for 15 min. Dinitrosalicylic acid reagent (3 mL) was then added and the reaction mixture was boiled in water bath for 15 min. Immediately after boiling, 1 mL sodium potassium tartrate (40% w/v) was added into the mixture for colour stability. The mixture was cooled in a water bath to room temperature and the absorbance of cooled reaction mixture was measured at 540 nm (Jasco V-630 spectrometer) against a blank.

The standard curve was established using D-galacturonic acid as a reducing sugar. One unit (U) of polygalacturonase activity was defined as the amount of enzyme that releases 1µmol of galacturonic acid per min under the assay conditions. The enzyme activity was calculated by the formula; [20].

$$\text{Enzyme Activity} = \frac{\text{D-galacturonic acid } (\mu\text{mol/mL}) \text{ x Total solution volume (mL)}}{\text{Enzyme added (mL) x Reaction time (min)}}$$

2.4 Identification of the Selected Pectinolytic Bacterial Isolate

2.4.1 Microscopic observation and biochemical characterisation

The pectinolytic bacterial isolate which showing maximum enzyme activity was morphologically, microbiologically and biochemically characterized. These characterization tests include; colony morphology, Grams reaction, spore formation, motility, catalase, oxidase, urease, starch hydrolysis, nitrate reduction, carbohydrate fermentation tests and IMVIC tests [21,22].

2.4.2 Identification of the microbial culture using 16S rDNA based molecular technique

DNA isolation from pure isolate was done using XcelGen bacterial gDNA kit (Cat # XG2411-01) as per protocol recommended by manufacturer. The universal16S rDNA primer 8F and1492R were used for amplification of genomic DNA by polymerase chain reaction (PCR). The PCR and agarose gel procedures were done as described by Sing et al. 2013 [23]. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The amplified product was excised from the gel and purified using XcelGen DNA Gel/PCR Purification Mini Kit (Xcelris Labs Limited, India). The concentration of the purified DNA was determined and the 16S PCR product of the isolate was sequenced in both directions. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BigDye Terminator v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyser (Applied Biosystems, USA) following manufacturer's instructions mentioned in the kit.

2.4.3 Sequence analysis and phylogenetic tree construction

Consensus sequence of 1427 bp of 16S region was generated from forward and reverse sequence data using aligner software. The 16S region sequence was used to carry out BLAST with the nr database of NCBI genbank. Multiple sequence alignment was performed by using CLUSTAL W [24]. These alignment results of the first ten high similarity sequences were selected along with the unknown sequence to construct phylogenetic tree using MEGA 5.0 software tool [25]. Kimura two-parameter method [26] was used to compute the evolutionary distances and the codon positions included were 1st+2nd+ 3rd+Noncoding. The phylogenetic tree was inferred using the neighbour-joining methods [27]. Bootstrap analysis was based on 500 re-samplings.

2.5 Exo-pectinase Production

Further enzyme production studies by the identified isolate was done in modified yeast extract pectin (YEP) broth containing (g/l) Citrus pectin 2.5, yeast extract 5.6 g, K_2HPO_4 0.3 g, $CaCl_2$ 0.4 g with pH 7. Batch mode shake flask experiments were conducted at 30ºC and 150 rpm for 24 h in 250 mL Erlenmeyer flasks containing 50 mL of the media inoculated with 1% of inoculum. The fermentation broth was then centrifuged at 10000 rpm for 10 min, and the cell-free supernatant was used for various analysis.

2.6 Effect of Incubation Time on Pectinase Production

Effect of incubation time on enzyme production was studied at different time intervals in 30°C at 150 rpm. The enzyme assay was performed at every 12 h incubation period.

2.7 Types of Exo-pectinase Activity

Different methods as described by Tewari et al. 2005 [28] were used to detect the types of exo-pectinase produced by the bacterial isolate in 30ºC at 150 rpm for 72 h.

2.7.1 Polygalacturonase (PG)

PGase activity was assayed by DNS method (as discussed in 2.3). The substrate used here was 0.5% (w/v) polygalacturonic acid (Himedia).

2.7.2 Pectin lyase (PNL)

The PNL activity of the strain was assayed by thiobarbituric acid (TBA) method as described by Pitt., 1988 [29] with minor modification. The cell-free supernatant (0.1 mL) was added to 0.5 mL of the pectin solution (1% w/v). Test sample volume was then adjusted to 1.0 mL with Tris-HCl buffer (0.02 M, pH 8). The samples were incubated at 40ºC for 1 h. To this, zinc sulfate (0.06 mL, 9.0% w/v) and sodium hydroxide (0.06 mL, 0.5 M) was added. The samples were centrifuged at 3000 rpm for 10 min and 0.5 mL of the clear supernatant was added to a mixture of TBA (0.3 mL, 0.04 M), HCl (0.25 mL, 0.1 M), and

distilled water (0.05 mL). The mixture was then heated in a boiling water bath for 30 min, cooled to room temperature, and the absorbance of the colored solution was measured at 550 nm against a reference cuvette which contained the same reagents as that of the experimental cuvette but for which the zinc sulphate and sodium hydroxide were added before adding the enzyme and substrate. The amount of enzyme that caused a change in absorbance of 0.01 under the condition of the assay was defined as 1 unit (U) of activity.

2.7.3 Pectate lyase (PEL)

PEL was assayed in the similar procedure as mentioned above (in 2.7.2) for pectin lyase. The substrate used here was 0.5% (w/v) polygalacturonic acid instead of pectin. The occurrence of a peak at 550 nm was due to the hydrolytic products formed by pectate lyase. The amount of enzyme that caused a change in absorbance of 0.01 under the condition of the assay was defined as 1 unit (U) of activity.

2.8 Partial Purification of Pectinase

Partial purification of pectinase from the isolate was done from 100 mL modified YEP culture broth (growth conditions: 30°C, 150 rpm, 72 h) by ammonium sulphate precipitation. The cell-free supernatant was saturated with $(NH_4)_2SO_4$ to two cut-offs (0 - 40%, 40 – 100% saturation). The precipitates were dissolved in minimum amount of Tris-HCl buffer (0.02M, pH 8) and dialysed against the same buffer [5].

2.9 Detection of Biosurfactant Activity

2.9.1 Foam formation activity

The bacterial strain was cultured separately in 100 mL Erlenmeyer flask containing 20 mL modified YEP broth. The flask was incubated at 30°C on a shaker incubator (150 rpm) for 72 h. Foam activity was detected based on duration of foam stability, foam height and shape in the graduated cylinder [30].

2.9.2 Microplate assay

For microplate assay, 100 µl of culture supernatant was taken in a well of a 96 – microwell plate. The well with supernatant was watched by means of a backing sheet of paper with grid. If biosurfactant is present, the concave surface distorts the image of grid below and it provides a qualitative assay for the presence of surfactants [31].

2.9.3 Drop collapse and oil displacement test

The drop collapse qualitative test according to Youssef et al. [32] was done. To a solid surface 2 µl mineral oil was added and equilibrated for 1 h at room temperature. To this oil surface, 5 µl culture supernatant was added and inspected after 1 min. In oil displacement test, 15 µl of crude oil was placed as a film on the surface of distilled water (40 µl) in a Petri dish. Over the oil layer, 10 µl of culture supernatant was poured and the clear halo under visible light visualized were noticed [33].

2.9.4 Penetration assay

The cavities of a 96 – microwell plate were filled with 150 µl of a hydrophobic paste consisting of oil and silica gel. The paste was covered with 10 µl of oil. Then 90 µl supernatant was coloured with 10 µl of a red staining solution (safranin). The coloured supernatant was then placed on the surface of paste. If biosurfactant is present, the hydrophilic liquid will break through the oil film barrier into the paste [34].

2.9.5 Hemolysis

Biosurfactants can cause lysis of erythrocytes. This principle is used for hemolysis assay which was developed by Mulligan et al. 1984 [35]. Culture supernatant inoculated on blood agar plates was incubated for 1 - 2 days at 30°C. A colourless or clear zone indicated a positive result.

2.9.6 Emulsification activity (E_{24})

To measure emulsification capacity, 2 mL of different oils (kerosene, engine oil, diesel and petrol) were added to equal volume of culture supernatant and the mixture was vortexed at a high speed for 2 min. After 24 h, the height of the stable emulsion layer was measured. Uninoculated medium was used as control. The emulsion index was calculated as the ratio of the height of the emulsion layer and the total height of the liquid [36]. The emulsification activity of the crude extract was compared with tween 80 and paraffin.

2.9.7 Bacterial adhesion to hydrocarbon assay (BATH)

A turbid aqueous suspension (2 mL) of washed microbial cells was mixed with 2 mL of

hydrocarbon (kerosene). After mixing for 2 min, the two phases were allowed to separate. Hydrophobic cells become bound to hydrocarbon droplets and rise with the hydrocarbon. They were removed from the aqueous phase. The turbidity of aqueous phase was measured at 600 nm. The decrease in the turbidity of the aqueous phase correlates to the hydrophobicity of the cell. The percentage of the cells bound to the hydrophobic phase (H) was calculated by the following equation [37]: $H = (1- A/A_0) \times 100\%$ where, A_0 and A were A600 before and after mixing with hydrocarbon, respectively.

2.10 Isolation of Biosurfactant

A combination of acid and solvent extraction procedure [38] was followed to isolate biosurfactant produced from the strain with minor modification. Bacterial cells were removed from the surfactant-containing medium by centrifugation (10000 rpm for 20 min at 4°C). The surfactants were precipitated from the supernatant by adding 6 N HCl to obtain a final pH of 2.0 and allowed to stand at 4°C for 16 h. The precipitate was then centrifuged at 10000 rpm for 20 min at 4°C. Precipitate was collected and further extracted with methanol by keeping in shaker at 200 rpm for 5 h. The surfactant containing methanol was collected after filtration. The extract was neutralized immediately to avoid formation of methyl esters.

2.11 Statistics

All assays were conducted in triplicate and the values were given as mean ± SE. Graphs were draw by Microsoft Excel. Error bars in the graph represent standard error of the mean (SEM). Adobe Photoshop CS5 and Gimp image editor were used for setting the images.

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening of Bacterial Isolates for Pectinolytic Activity

A number of bacterial strains were isolated from the collected soil and other samples in YEP media by spread plate method. From these isolates, effective pectinase producers were screened by checking the substrate utilization zone around the inoculated colonies with the aid of iodine solution. Thirty six bacterial isolates are thus screened from this preliminary plate assay. These thirty six cultures were subjected to agar well diffusion assay to measure the substrate utilization zone formed by them. In agar well diffusion assay, four isolates with pectinolytic activity were selected on the basis of zone size ranging from 20 mm to 26 mm after flooding the plate with iodine solution (Fig. 1). The zone formation in agar well was also confirmed by adding CTAB.

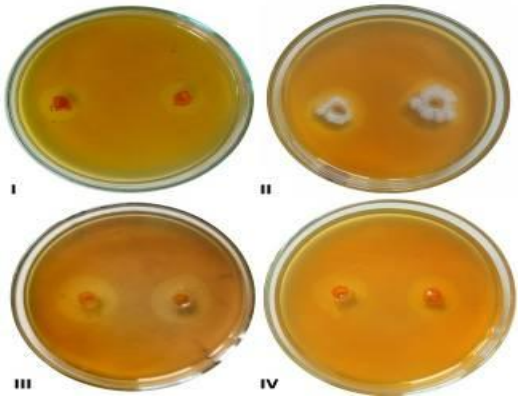

Fig. 1. Bacterial cultures showing pectin depolymerisation zones in agar well diffusion assay

All the four bacterial samples which were selected according to pectin depolymerisation zone in well diffusion assay were subjected to spectrometric assay by DNS method. The bacterial isolate, which showed maximum enzyme production in DNS assay (0.707 U/mL), was the same strain which exhibited larger pectin depolymerization area (26 mm) in well diffusion assay. This bacterium was labelled as isolate BKDS1 and further characterization and identification was carried out.

3.2 Morphological, Microbiological and Biochemical Characterization of the Isolate

The colonies of selected bacterial isolate BKDS1 were larger and the margin was undulate with circular form and flat elevation. It can able to grow in 6.5% NaCl and it was rod-shaped, Gram-positive motile bacterium producing endospore. This isolate had shown positive test for catalase, nitrate reduction, starch hydrolysis, Voges-Proskauer, citrate utilization and can able to ferment glucose and sucrose. Whereas, negative for urease, oxidase, hydrogen sulfide production, indole production, methyl red and unable to ferment lactose, mannitol and arabinose. The results obtained were compared with identification flowchart of Bergey's Manual [21]. The characteristics showed by this organism were fairly similar to *Bacillus subtilis.*

3.3 Identification on the Basis of Phylogenetic Analysis

The phylogenetic tree generated using 16S rDNA gene sequences of the bacterial isolate showed that the bacterium has the highest homology (99%) with *Bacillus subtilis* (GenBank Accession Number: AB042061.1) and designated as *Bacillus subtilis* BKDS1. Fig. 2 signifies phylogenetic tree of the isolate BKDS1 with the selected best homologous known bacterial strains. The tree was inferred by the neighbour joining method using the MEGA 5 software. Numbers at nodes of the tree are indications of the levels of bootstrap support based on a neighbor-joining analysis of 500 inferred replicates. The partial 16S rRNA gene sequences of the isolate *B. subtilis* BKDS1 have been deposited in the NCBI nucleotide sequence database under the accession number KT004506.1

3.4 Effect of Incubation Time on Pectinase Production

Optimal incubation time for maximal pectinase activity of isolated *B. subtilis* was found to be 72 h (Fig. 3). The enzyme production increased in 60 h (1.278 U/mL) and reaches maximum at 72 h (1.288 U/ml). Recently, Paudel et al. 2015, reported 72 h to be optimum incubation time for maximal PGase activity in *Bacillus* sp. HD2 [39]. Furthermore, Jayani et al. [40] also recounted similar finding supporting this result.

3.5 Types of Exo-pectinase Activity

Pectinase activity in cell free filtrates was assayed spectrometrically by different methods. The assay result revealed that the organism produces enormous amount of exo-pectinase enzymes. The polygalacturonase activity was assayed by the quantification of reducing sugars using DNS method and the unit enzyme activity was found to be 1.688 U/mL. Pectin lyase and pectate lyase activity was assayed by TBA method. The enzyme activity obtained was 110.2 Units and 231.0 Units (Expressed in units, 0.01 OD change taken as 1 unit of enzyme activity under the conditions of assay) for pectin and pectate lyase respectively. From this result, it is clear that the isolated strain of *B. subtilis* BKDS1 can exhibit different types of exo-pectinase activity. Previous reports of Kashyap et al. [5] and Soares et al. [41] supported this result.

3.6 Partial Purification of Pectinase

The partial purification of pectinase was done by ammonium sulphate precipitation and the active fraction was concentrated by dialysis. The enzyme activity was found in the 40 - 100% salt saturation fraction. Previous work on *Bacillus sp*. DT7 by Kashyap et al. [5] also presented similar result.

Isolation of microbial strains exhibiting innovative properties of enzymes with desirable

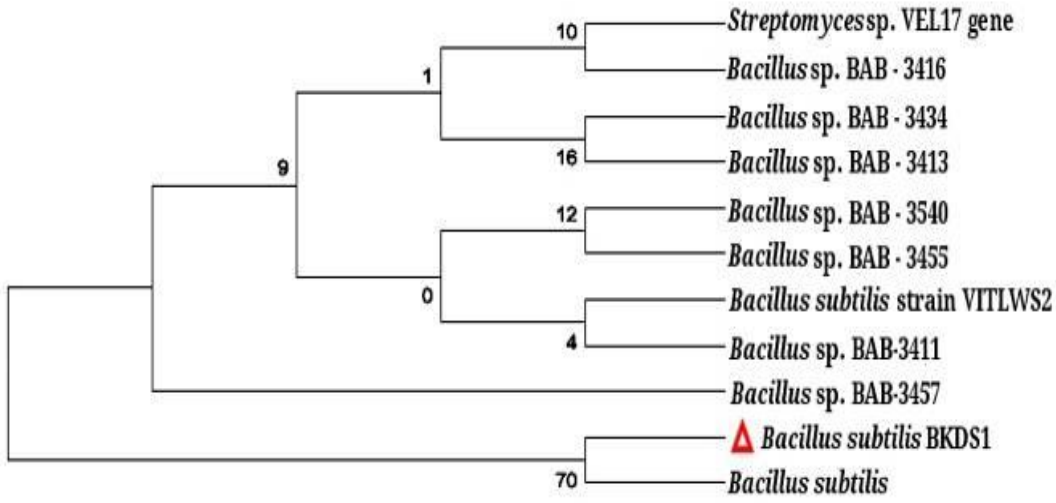

Fig. 2. Phylogenetic tree of the isolate *Bacillus subtilis* BKDS1 with the selected best homologous known bacterial strains

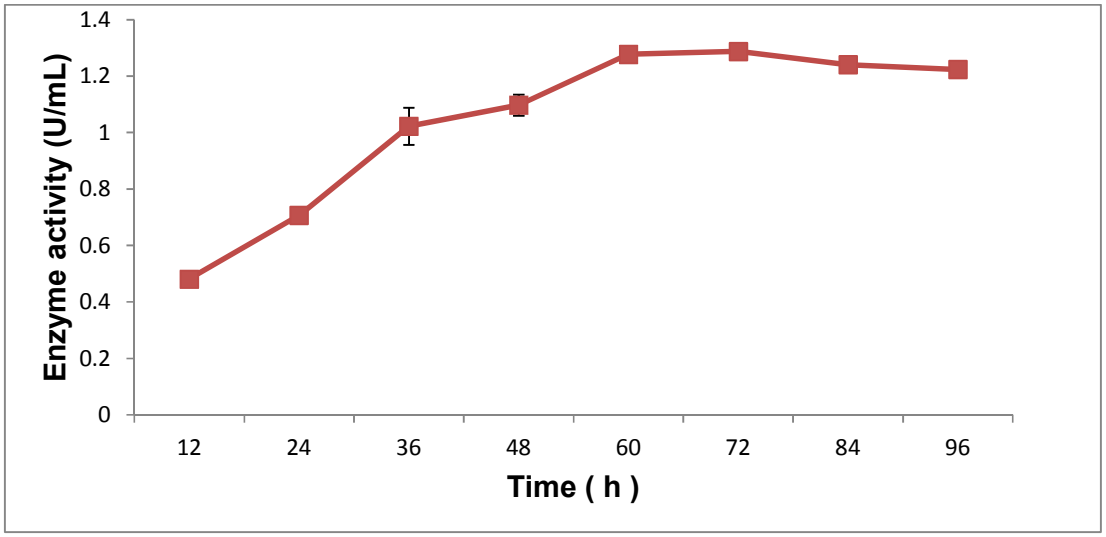

Fig. 3. Effect of incubation time on pectinase production

characteristics and low cost is always in demand for fulfilling national necessity and to upsurge industrial economy.

Bacillus subtilis is one of the most versatile of bacteria which was reported as a potent strain for various enzyme productions including pectinase. Findings regarding isolation of pectinase producing strains with novel properties are reported recurrently. Recent publications [7,39,42] by various authors supported the intention of this study.

3.7 Detection of Biosurfactant Activity

Biosurfactant detection in the culture supernatant was carried out by various primary screening methods such as foam formation, drop collapse assay, oil displacement test, emulsification activity, microplate assay, hemolysis, penetration assay and BATH assay. All of these methods showed positive results.

Foam produced in the culture broth by the organism after 72 h of incubation (Fig. 4A) was the indication for the presence of biosurfactant. The height of the foam was 2.5 cm and the round shaped foam was stable for 1- 2 days. Foam formation by the culture is considered as the primary indication for biosurfactant production [29].

3.7.1 Microplate and penetration assay

Surface activity of culture supernatant was determined qualitatively by microplate assay. The culture supernatant showed optical distortion. The concave surface distorted the image of the grid below indicated the presence of surfactants (Fig. 4B). This assay was rapid, easy and sensitive method which allows direct detection of surface active compounds. In penetration assay, the test sample was broken through the oil film barrier into the paste (Fig. 4C1). The silica gel entered to the hydrophilic phase and the upper phase changed from red to cloudy white (Fig. 4C2) which resulted in mixing of the two distinct phases within 15 min. This assay relies on the phenomenon that silica gel is entering the hydrophilic phase from hydrophobic paste much more quickly if biosurfactants are present. Recent work by Sing and Sedhuraman [43] supported this study.

3.7.2 Drop collapse test and oil displacement test

The drop collapse test and oil displacement tests are suggestive of the surface and wetting activities [44]. Drop collapse test and oil displacement test were highly positive for culture supernatant of tested bacterial strain. In drop collapse test, the culture supernatant gave flat drops over oil coated solid surface very quickly. Uninoculated media and distilled water was tested as control and showed no change (Fig. 4D). This assay has been applied several times for screening purpose. But it seems a low sensitivity since a significant concentration should be present to cause a collapse of aqueous drops on oil coated surfaces. The drops spread or collapsed because the force or

interfacial tension between the liquid drop and the hydrophobic surface was reduced. In oil displacement test, culture supernatant placed on the centre of the oil layer displaced the oil and a clear zone of approximate size 38 - 40 mm was formed (Fig. 4E). This was supported by Yeussef et al. [32] who adopted the technique to detect biosurfactant production by diverse microorganisms.

3.7.3 Hemolytic assay

The strain also showed positive result for hemolytic assay. The cell free supernatant caused lysis of erythrocytes which observed as clear zone around wells (Fig. 4F). Hemolytic assay is a commonly used method to screen biosurfactant production and in some works, it is the exclusive method used to monitor biosurfactant production [45]. But this method has some limitations also, such as lytic enzymes can led to clearing zones and non-diffusible surfactants can inhibit the formation of clearing zones [34].

3.7.4 Estimation of emulsification index (E_{24})

The culture supernatant showed emulsification as shown in Fig. 5. The activity was analysed with different oils and emulsification index was measured. Emulsification assay is an indirect method used to screen biosurfactant production. It was assumed that if the cell free culture broth used in this assay contains biosurfactant, then it will emulsify the hydrocarbons present in the test solution. In this assay the maximum emulsification index was showed with kerosene (75%), followed by diesel (60%) and engine oil (42.5%). Petrol showed least emulsification index (33.5%). Paraffin oil and tween 80 showed lesser E_{24} as compared with culture supernatant. They showed E_{24} activity of 40% and 15% respectively. In a recent work on biosurfactant production by *B. subtilis,* the highest emulsification was obtained with kerosene (46.90%) [46].

3.7.5 Bacterial adhesion to hydrocarbon assay (BATH)

Hydrophobicity of cells is considered as an indirect method to screen bacteria for biosurfactant production, because cells attach themselves with oil droplets by producing surface active compounds called biosurfactants. The hydrophobicity of *B. subtilis* obtained in this BATH assay was 52.7%. This is an indication for the affinity of the bacterial cells towards hydrophobic substrate. In some studies, BATH assay was considered as the principal method for screening of biosurfactant production [47].

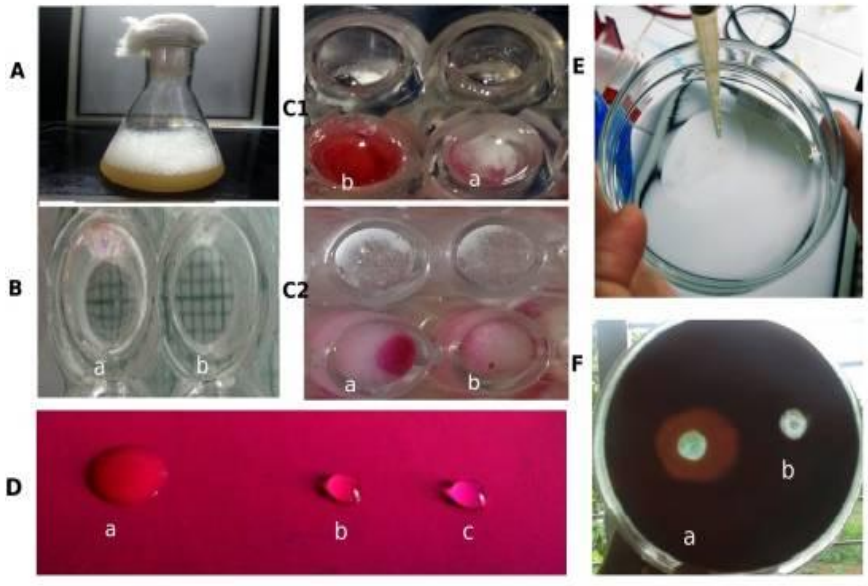

Fig. 4. A. Culture showing Foam formation B. Microplate assay (a. Test - culture supernatant b. Control – distilled water). C. Penetration assay -C1 Upper phase & C2. Bottom phase (a. Test b. Control) D. Drop collapse Test (a. Test - culture supernatant b. Control - Uninoculated medium c. Distilled water) E. Oil Displacement test F. Hemolytic assay (a. Test b. Control)

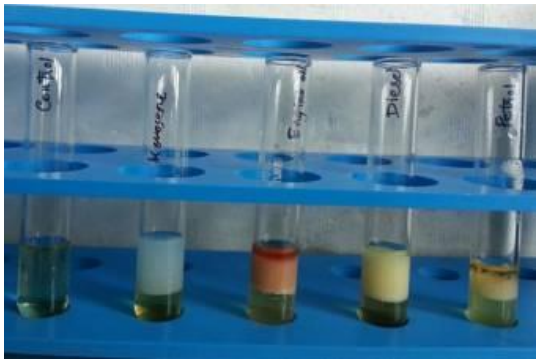

Fig. 5. Estimation of emulsification activity

3.8 Isolation of Biosurfactant

A light brown residue was obtained after the solvents were evaporated. A maximum concentration of 1.45 g/L was achieved at 72 h.

All the screening methods executed to investigate the biosurfactant producing capability of the isolated strain *B. subtilis* BKDS1 showed positive results. So, the ability of this strain to produce biosurfactant in pectinase production medium was confirmed. Simultaneous production of various enzymes with biosurfactant was reported previously [14-16]. But no reliable reports are available regarding simultaneous production of pectinase and biosurfactants. These findings thus, corroborate the design of concurrent production of these products with the isolated strain *B. subtilis* BKDS1.

4. CONCLUSION

Advancement in industrial, agricultural and biotechnological fields, have resulted in the need for search of microorganisms with novel characters that can be used for scientific and industrial purposes. These products include enzymes, polyunsaturated fatty acids, antimicrobials, biosurfactants, exopolysaccharides, probiotics, bacteriosins etc. In this study, the newly isolated strain of *Bacillus subtilis* BKDS1 was found to produce pectinase and biosurfactant in the same culture media.

Optimal incubation time for maximal pectinase activity of isolated *B. Subtilis* BKDS1 at 30°C was found to be 72 h. DNS assay for polygalacturonase and TBA assay for pectin lyase and pectate lyase were performed to check the potential of the strain to produce various exo-pectinase enzymes. The enzyme was partially purified by ammonium sulphate precipitation (40-100% salt saturation) and followed concentration by dialysis.

The biosurfactant production was screened by various methods like foam formation, drop collapse test, oil displacement test, microplate assay, hemolytic assay, penetration assay, emulsification activity and BATH assay. These entire screening tests showed positive results and thus the biosurfactant production was confirmed.

Our results indicate the possibility of an integrated process for obtaining pectinase enzymes and biosurfactants in the same culture media. So this could greatly increase the economic viability of this strain. Use of cheap culture media components such as agro-wastes, optimization, product enhancement and application are the future prospects of this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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