Biomaterials and Medical Applications

Research Article

Distribution of Antimicrobial ε-polylysine Producing Marine Microbe in Sea Water along West Coast of India

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Abstract

Crude glycerol is an inexpensive waste material of biodiesel manufacturing industries. The current demand is to explore useful products such as ε-Polylysine from crude glycerol (CG). In this present research, *Bacillus licheniformis* PL26 was found to produce 155 mg ε-Polylysine l^{-1} from the feed containing 2% crude glycerol and 93ml M3G medium after optimizing upstream process parameters (Graphical abstract).
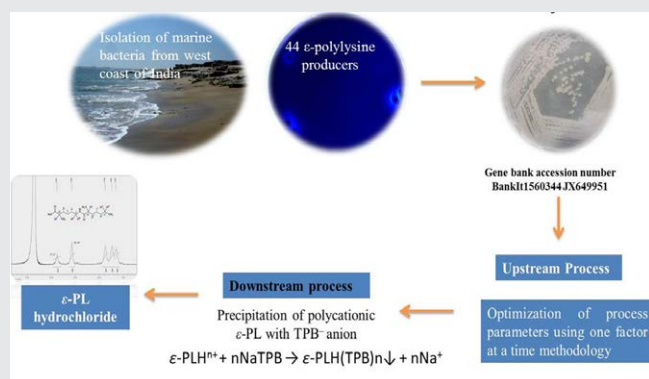
Keywords

ε-Polylysine; Crude Glycerol; *Bacillus licheniformis*; Optimization; NaTPB

Introduction

ε-Polylysine (ε-PL) is a homopolymer of L-lysine linked by the peptide bond between the carboxyl and ε- amino groups [1,2]. ε-PL is water soluble, biodegradable, edible and non-toxic towards human and the environment. Hence, its derivatives have been used the past few years for a broad range of industrial applications such as food, medicine, environment and electronics [3]. ε-PL exhibits antimicrobial activity against a wide spectrum of microorganisms, including Gram-positive and Gram-negative bacteria [4]. Furthermore, due to its

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antimicrobial and biodegradable nature, ε-PL has been introduced as a safe food preservative. In Japan, it has already entered the commercial market and is produced industrially by fermentation method using a mutant derived from *S. albulus* [5]. ε-PL and its derivatives also offer a wide range of unique applications such as emulsifying agents, dietary agents, biodegradable fibers, highly water absorbable hydrogels, drug carriers, gene delivery carriers, anticancer agent enhancer, biochip coatings, micro or nano capsules for delivery of ocular drug, encapsulation of cell lines for delivery of bioactive molecules *in vivo* [6-20].

Worldwide, there is a huge demand of the transportation fuels on the other hand there is access to petroleum oil reserves. Hence, there is more emphasis on renewable and alternative resource of energy to cope with ever increase demand [21]. Moreover, research reveals that biodiesel, is one of the promising alternative and renewable fuels, has been viewed with increasing interest as well as its production capacity has been well developed in recent years, however, researchers are exploiting various ways of utilizing crude glycerol for value added products in order to reduce the production cost involved in biodiesel manufacturing process [22]. The main reason of its relatively high production cost of Jatropha biodiesel as compared to conventional fossil fuel is the cost of plantation, which can be further reduced through utilization of the crude glycerol (co-product of biodiesel process) [22]. It was projected that the world biodiesel market would reach 37 billion gallons by 2016, which implied that approximately 4 billion gallons of crude glycerol would be produced [22]. In the synthesis of biodiesel, oils and fats are transesterified to fatty acid methyl ester in the presence of sodium hydroxide or potassium hydroxide. Hence, glycerol is generated as stoichiometric byproduct with a ratio of 10 % (w/w) with respect to biodiesel produced that cannot be used for food grade applications or other applications due to its poor quality. Basically this material could be valuable low cost carbon source and renewable material for synthesis of ε-Polylysine (ε-PL).

One of the major problems being faced by the researchers during increasing ε-PL production in crude glycerol is low oxygen transfer rate as glycerol restrains the oxygen transfer rate and reduces the growth rate. One of the wild strains of *Streptomyces albulus* (CCRC 11814) is having the capability of utilizing crude glycerol (byproduct of biodiesel process) for production of ε-PL [22]. ε-PL production can also be done from glucose derived from molasses using *Bacillus sp.* [23], However, addition of 10 g/l glucose at 12h interval followed by addition of 5 mM citric acid after 24 h and 2 mM L-aspartic acid after 36 h, resulted in 0.565 g/l ε-PL yield from *Bacillus cereus* having 36h production age [24].

Another major challenge being faced is the downstream processing for the purification of ε-PL from fermentation broth containing crude glycerol. Researchers made various approaches for purifying ε-PL in a cost effective manner. Cation exchanger (Amberlite IRC-50) column adsorption is one of the methods for ε-PL extraction from fermentation broth [25]. The drawback of the column adsorption process is that ε-PL fermentation broth containing crude glycerol needs prior clarification before loading into the column in order to avoid clogging in the column.

Another approach being reported for cost effective downstream

processing for recovering polycationic ε-PL from fermentation broth using *Streptomyces albulus* is using polyanionic magnetic nanoparticle (MNP). Polyanionic MNP was prepared by co-precipitating Fe (II)/ Fe (III) solution in presence of water soluble carboxymethyl cellulose (CMC) at pH 11.0 for adsorbing ε-PL generally having up to 80 % adsorption capacity. The CMC matrix can be recycled up to five times [26].

Purification of ε-PL can be done directly from the fermentation broth in the form of ε-PL hydrochloride through precipitating it with tetraphenylborate anion. At pH 3.5, ε-PL-Hn^+ cation associates with the TPB^- anion to form a precipitate of 1: n stoichiometry, ε-PL-H $(TPB)_n$ [27], but at the same time other monovalent cations will also be precipitated with TPB^-. However, washing the precipitate with acetone will remove the NH_4TPB and KTPB which are more soluble in it and finally ε-PL can be precipitated as hydrochloride salt by mixing ε-PLH $(TPB)_n$ precipitate with HCl (10%) solution [28].

In the current context, bacterial isolates from marine water samples from west coast of India were primarily screened on agar plates containing basic dye methylene blue. The bacterial strain giving maximum ε-PL content was further considered for medium optimization using one factor at a time methodology for optimizing the process parameters (carbon source concentration, pH and production age). Furthermore, the downstream processing for purifying ε-PL was carried by ammonium sulphate precipitation, followed by precipitating it through reacting with sodium tetraphenylborate.

Experimental

Study sites and collection strategy

The study sites (Figure 2) were located in west coast of India having geographical locations as mentioned in Table 1. The sea water samples were collected during low tide from Jafarabad, Chakratirth, Adri, Nagoa Beach (Diu), Veraval, Madhavpur, Okha coast, CSIR-CSMCRI's experimental salt farm, Vector port, Koteshalay Jetty, Jakhau port, Mandavi temple, Mundra port, Mandavi beach, along the west coast of India (Tables 2 and 3).

Sea water analysis

The physicochemical parameters of water such as pH, temperature, salinity, total dissolved solids (TDS) and dissolved oxygen (DO) were analyzed using pre-calibrated multi-parameter probe (Thermofischer, USA) at the site.

Isolation of bacteria

Ten-fold dilutions of the sediment samples were prepared and 0.1 ml from each sample solution was poured on Zobell marine agar

Table 1: Geographical location of the sites having ε-polylysine producing bacteria.

Sites	Latitude	Longitude	pH	Temperature	
				Air	Water
Bhavnagar (CSMCRI Experimental Salt Farm)	21°47'23"N	72°7'47"E	7.8	35°C	29°C
Gopnath Sea Coast	21°20'98'N	72°10'97'E	7.9	32°C	29°C
Rajula (Victor Port)	20°94'144'N	71°57'93'E	8.1	32°C	29°C
Jafarabad Sea Coast	N 20°51.547'	71° 22.876' E	8.2	34°C	30°C
Diu (Nagoa Beach)	N 20° 42.412'	70° 54.950' E	8.3	32°C	29°C
Veraval	N 200 58.593'	70°16.761' E	6.8	30°C	29°C
Adri	N 20° 57.593'	70°16.767' E	7.9	30°C	28°C
Junagarh (Madhavpur Beach)	N 21°16.303	69°56. 96 E	7.1	21°C	26°C
Kutch (Mandvi Beach)	N22°49.292"	69°20.66" E	7.86	32°C	30°C

Table 2: Quality of seawater dominant with ε-polylysine producing bacteria along the west coast of India.

Sites	DO (mg/l)	Salinity (g/Lit.)	TDS (mg/lit.)
Bhavnagar (CSMCRI Experimental Salt Farm)	7.76	35	81196
Gopnath Sea Coast	8.03	35	27502
Rajula (Victor Port)	6.6	27.74	28931
Jafarabad Sea Coast	5.6 ± 0.028	35	40280 ± 1.96
Diu (Nagoa Beach)	7.76 ± 0.58	39.11 ± 6.88	55304
Veraval	4.02	30	26801
Adri	4.26	34.9	35105
Junagarh (Madhavpur Beach)	3.048	35	27101
Kutch (Mandvi Beach)	8.13	35.77	26800

**Table 3:** Percentage carbon utilization of *Bacillus licheniformis*.

Parameter	Concentration
Biodiesel waste residue (crude glycerol)	10 g
pH	6.0
Total Carbon content in fermentation medium	0.7 g
Total Carbon left in the supernatant	0.4047 g
Carbon present in the biomass	0.21
Total volume	100 ml
Percentage carbon utilized (%)	52.11

plates containing (g/l) peptone 5.0; yeast extract 1.0; ferric citrate 0.1; sodium chloride 19.45; magnesium chloride 8.8; sodium sulfate 3.24; calcium chloride 1.8; potassium chloride 0.55; sodium bicarbonate 0.16; potassium bromide 0.08; strontium chloride, 0.034; boric acid, 0.022; sodium silicate, 0.004; sodium fluoride, 0.0024; ammonium nitrate, 0.0016; disodium phosphate, 0.008; agar, 1.5, at pH 7.6 ± 0.2. The plates were incubated at 37°C temperature for 48 h.

Screening of bacteria

A novel and faster screening was implemented on the basis of growth and ε-PL content. Screening was carried out by streaking bacterial isolates on agar plates containing basic dye methylene blue [29]. The growth medium containing (g/l) glycerol 10, ammonium sulphate 1.0, disodium hydrogen phosphate 0.5, magnesium sulphate 0.25, yeast extract 0.5, potassium dihydrogen phosphate 0.5, agar 2%, and methylene blue 0.02% were maintained at pH 7.0. The isolated bacterial strains were streaked on the plates containing methylene blue as the basic dye and kept at incubation period of 168 h at 32°C. The growth of microorganism producing extracellular basic polymers such as ε-PL on agar plates containing methylene blue dye shows an outer zone which was later confirmed by adding the biomass of ε-PL producing strain as well as standard ε-PL (100 mg) in the wells of the plates containing the 0.02 % basic dye (methylene blue) (Figure 1a and 1b). 44 isolates (PL 1-44) were obtained which showed non-uniform zone distribution on the methylene blue plates.

The bacterial isolates which showed growth on methylene blue medium, was further sub-cultured in a flask containing 100 ml of M3G production medium. For production, 500 ml of Erlenmeyer flask containing 100 ml of production medium (M3G) composed of (g/l) biodiesel waste residue 5, yeast extract 1.5, $MgSO_4.7H_2O$ 0.05, $FeSO_4.H_2O$ 0.003, $ZnSO_4$ 0.004, KH_2PO_4 0.136, Na_2HPO_4 0.358, prepared in the distilled water. The pH was maintained at 6.0 ± 0.2. The 24 h of log culture of individual strains was developed for preparing the inoculum. 5% inoculum of the seed culture was inoculated in the production medium and was kept for 120 h incubation in rotatory
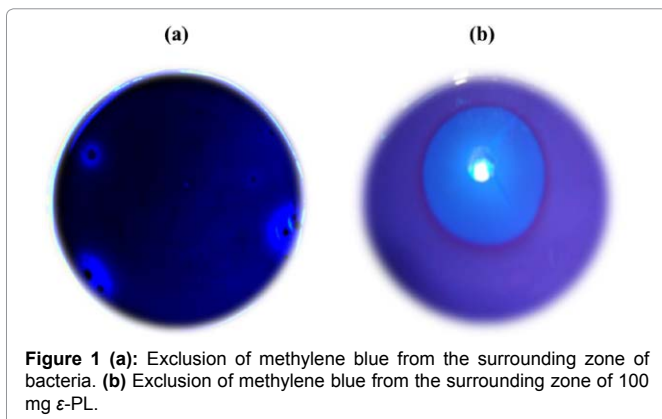
Figure 1 (a): Exclusion of methylene blue from the surrounding zone of bacteria. **(b)** Exclusion of methylene blue from the surrounding zone of 100 mg ε-PL.

shaker at 30ºC and 120 rpm. After 120 h, the broth was centrifuged at 9,000 rpm and the supernatant was analysed quantitatively for the ε-PL production by using the trypan blue precipitation method [30].

Fermentation

Culture media: The strain was cultivated in marine medium containing (g/l) peptone 5.0; yeast extract 1.0; ferric citrate 0.1; sodium chloride 19.45; magnesium chloride 8.8; sodium sulfate 3.24; calcium chloride 1.8; potassium chloride 0.55; sodium bicarbonate 0.16; potassium bromide 0.08; strontium chloride, 0.034; boric acid, 0.022; sodium silicate, 0.004; sodium fluoride, 0.0024; ammonium nitrate, 0.0016; disodium phosphate, 0.008. The media was adjusted to pH 7.6 ± 0.2 the plates were incubated at 37ºC temperature for 48 h.

Inoculum: Isolated *Bacillus licheniformis* PL26 was first isolated from slant (solid medium) grown for 24h. The seed culture was incubated overnight in 250 ml shake flask containing 100 ml of the medium at 30°C for *Bacillus licheniformis* PL26, and with a pH value of 7.0 in an incubator shaker at 120 rpm. Crude glycerol (20 g/l) was used in the preculture with *Bacillus licheniformis* PL26 to preadapt it to carbon source. The inoculum for the production batch was prepared from loopful of *B. licheniformis* streaked on slants, which was then transferred to 250 ml shake flask containing 100 ml media and incubated under the same conditions as seed culture.

Production of ε-PL: ε-PL was produced by cultivation of *Bacillus licheniformis* in a two stage process through submerged fermentation in shake flask scale (100 ml). In the first stage, a loopful of *Bacillus licheniformis* inoculated to seed culture medium containing (g/l) peptone 5.0, yeast extract 1.0, ferric citrate 0.1, sodium chloride 19.45, magnesium chloride 8.8, sodium sulfate 3.24, calcium chloride 1.8, potassium chloride 0.55, sodium bicarbonate 0.16, potassium bromide 0.08, strontium chloride 0.034, boric acid 0.022, sodium silicate 0.004, sodium fluoride 0.0024, ammonium nitrate 0.0016 and disodium phosphate 0.008 and then cultivated for 48 h at 30ºC for getting the dense seed inoculum appropriate for inoculating into production medium. In the second stage, 5% of the seed culture having 2.42 optical density inoculated to production medium containing (g/l) ammonium sulphate - 10.0, potassium dihydrogen ortho phosphate - 1.36, dipotassium hydrogen phosphate - 0.8, magnesium sulphate - 0.5, ferrous sulphate - 0.04, zinc sulphate - 0.03, and biodiesel waste residue (BWR) - 20 and pH 6.0 ± 0.2. The production batch was kept at 30ºC in an incubator shaker at 220 rpm for 120 h.

ε-PL assay: After completion of the fermentation, the culture was centrifuged (9000 rpm, 10 min), and the ε-PL concentration was measured in the supernatant, which is based on selective binding of trypan blue by ε-PL [30]. Briefly, 1 ml of supernatant was added to 2.88 ml of 0.1 mM phosphate buffer (pH 7.0), 120 μl of trypan blue solution (1 mg/ml), mixed thoroughly and incubated at 37ºC for 60 min. Absorbance was measured after centrifugation (9,000 rpm , 10 min) at 580 nm on UV-vis spectrophotometer (Varian, USA). A standard curve was derived from measurements with known amounts (0-50 mg/l) of ε-PL. The slope obtained from an equation correlating the absorbance with concentration of ε-PL was -0.0654 (R^2 = 0.984).

Percentage carbon utilization of *Bacillus licheniformis*

Percentage carbon utilized by *Bacillus licheniformis* was calculated as

$$\text{Carbon utilization } (\%) = (\text{Total glycerol present in the medium } - \text{ Total unutilized glycerol}) \times 100$$

Glycerol estimation was carried out by using 'Waters Alliance' high performance chromatographic system equipped with RI detector (Waters 2414 model) and separation module (Waters 2695 model). Chromatographic separations were performed on an 'Aminex HPX-87H' column (300 × 7.8 mm) (Bio-Rad Laboratories, Richmond, CA) with a precolumn (30 × 4.6 mm) of the same stationary phase (DVB-S, hydrogen form). Isocratic elution at a flow rate of 0.6 mL/min was carried out using a mixture of 5mM sulfuric acid. Peak detection was made by keeping the cells of the RI detector at 30 °C. The samples were appropriately degassed, twice diluted with double-distilled water, filtered through a 'Whatman' 0.45-μm filter membrane (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, United Kingdom), and then injected (50-μL loop volume). Data were obtained and processed by using 'Waters EMPOWER' software (waters Corporation). Peak identification was carried out by spiking the sample with pure standards and comparing the retention times with those of pure compounds.

Optimization of upstream process parameters for improving ε-PL production

Effect of biodiesel waste residue concentration, pH and production age on ε-PL production was assessed by using incubator shaker. The fermentation was performed with 100 ml of broth in a 250 ml flask that was incubated at 30°C with a shaker rotation speed of 220 rpm. The impact of various concentrations (2%, 4%, 6%, 8%, and 10%) of biodiesel waste residue, at pH values (6.0-9.0) and at different production age (24h, 48h, 72h, 96h and 120h) on ε-PL production was assessed.

Characterization of crude ε-PL production using SDS PAGE

After completion of the fermentation, the culture was centrifuged (9000 rpm, 10 min), and the supernatant was concentrated to 10 ml and applied to SDS polyacrylamide gel electrophoresis.

The molecular weight of the protein was detected by using polyacrylamide gel electrophoresis (PAGE) to separate the proteins by electrophoresis through a continuous polyacrylamide gel as a support medium and sodium dodecyl sulphate (SDS) to denature the proteins. However, ε-PL is a polycationic with a high isoelectric point PI of 9.0, it will further aggregate with the anionic SDS.

Downstream processing of ε-PL

Precipitation of polycationic ε-PL with TPB⁻ anion from the supernatant: The protein precipitate was collected and salting out was carried through dialysis by using membrane of 5.0 KDa and tris HCl buffer (pH=7). The protein precipitate which was left after salting out was treated with sodium tetraphenylborate for precipitating ε-PL

as a polyelectrolyte salt with the TPB⁻ anion. The polycationic ε-PL salt with the TPB⁻ anion was further purified by washing the mixed precipitate with acetone to remove triphenylborane and benzene. Thereafter, the precipitate reacted with 1M HCl for obtaining ε-PL hydrochloride.

Results and Discussions

Isolation and screening for ε-PL producing bacteria

A total of 44 bacteria isolated from soil and sea water samples were used for further research investigation having ε-PL producing capacity, on basis of exclusion of methylene blue from the outer zone of the isolated colonies (possessing ε-PL producing potential) on the methylene blue agar plates as shown in the Figure 1a. In this current study, a parallel phenomenon was also observed in the Figure 2b when pure powdered ε-PL (positive charge substance) was applied to the agar plates containing 0.02% methylene blue. The possible reason may be due to strong electrostatic interaction between ε-PL (positive charge substance) and basic dye (negative charge) [29]. Utilizing the above discussed procedure, it was possible to examine numerous microbes in a single run through the interaction between the charged groups of secreted polypeptides by the microbe and basic dye methylene blue.

ε-PL was produced by cultivation of *Bacillus licheniformis* by using biodiesel waste residue (crude glycerol) as the carbon source through submerged fermentation with an initial concentration of 38.66 mg/l. The principle behind replacing pure glycerol by biodiesel waste residue as the carbon source is to add more economics to the biodiesel manufacturing process. ε-PL content present in other strains e.g. *Streptomyces albulus* (38.45 mg/l), *Streptomyces noursei* (38.27 mg/l*)* and *Streptomyces virginiae* (38.47 mg/l) by utilizing the biodiesel waste residue were also in the comparable range with respect to the marine bacterial isolate *Bacillus licheniformis* which was obtained from salt pan of CSIR-CSMCRI's experimental salt farm.

Distribution of ε-PL producing bacteria in sea water along west coast of India

West coast of India can be considered as important area for

Figure 2: Distribution of ε-PL producing bacteria in sea water along west coast of India.

collecting diverse marine microorganism's ε-PL producing capacity. In this attempt, 44 marine isolates (PL 1-44) from west coast of India was obtained. Figure 2 illustrates distribution of ε-PL producing isolates along west coast of India, 13 isolates from Bhavnagar coast, 2 isolates from Gopnath coast, 3 from Rajula (Victor Port), 1 from Jafrabad coast, 6 from Diu (Nagoa beach), 2 from Veraval coast, 3 from Adri coast, 3 from Madhavpur (Junagarh) and 5 from Mandvi Beach (Kutch).

Percentage carbon utilization of *Bacillus licheniformis*

Carbon utilization percentage of isolated *Bacillus licheniformis* is shown in Table 1. *Bacillus licheniformis* isolated from salt pan is having 52.11% total carbon utilization percentage as it utilizes 0.21g total carbon from 0.7g total carbon present in the production medium.

Optimization of fermentation media using one factor at-a-time method

Impact of biodiesel waste residue concentration on ε-PL production by *Bacillus licheniformis* is shown in Figure 3. The optimum concentration of biodiesel waste residue (BWR) for the production of ε-PL (44.21 mg/l) by *Bacillus licheniformis* was found to be 2%. On the other hand, repeating same batch with 2% pure glycerol as carbon source yielded 40.32 mg/l ε-PL. Also, the effect of supplementing solely 2% BWR, M3G medium containing 2% BWR as carbon source and M3G medium containing 2% pure glycerol on ε-PL production was studied. It was observed that flask containing positive control medium i.e M3G medium containing pure glycerol yielded 38.72 mg/l. On the other hand, medium containing solely BWR (excluding other components present in M3G medium) yielded 39 mg/l ε-PL; M3G medium containing 2% BWR yielded 39.13 mg/l. The possible mechanism for increase in ε-PL content may be increase in ε-PL synthetase activity in crude glycerol as compared to the pure glycerol [31]. Therefore, M3G medium containing 2% BWR as the carbon source was considered for further studies.

Effect of pH on ε-PL production by *Bacillus licheniformis* is shown in Figure 4. The maximum ε-PL yield of 80.1 mg/l was obtained when the initial pH of the production medium adjusted to 8.5. As per available literature, initial pH of 6.5-7.5 produces comparable ε-PL yield. In the present pH study in the production medium decreases from 8.5-4.0 after 24h-48h. However, the decrease in pH may be due to the enzyme activity desired for ε-PL synthesis, which catalyzes the polymerization of L-lysine. Lowering the pH might accelerate the polymerization from L-lysine to ε-PL, while increasing the pH might

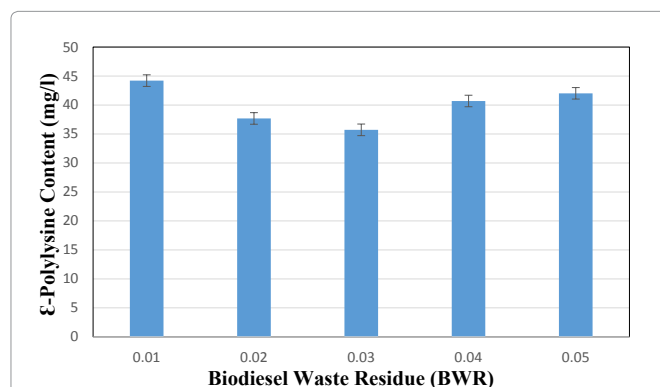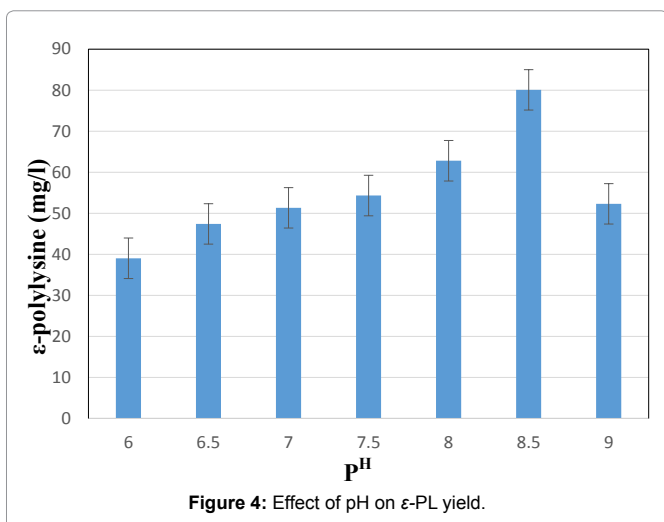**Figure 3:** Effect of various concentration of biodiesel waste residue (BWR) on ε-PL yield.

Figure 4: Effect of pH on *ε*-PL yield.

Figure 5: Effect of production age on *ε*-PL yield.

accelerate depolymerisation from *ε*-PL to L-lysine. This assumed mechanism is significantly related to the biosynthesis of other kinds of poly amino acids, such as gamma-poly (glutamic acid) production from *Bacillus licheniformis* [32].

This declination in pH supports *ε*-PL production which can be supported from the available literature [31], however, in most of the reports, at this pH bacterial growth gets ceased and if harvesting of fermentation broth is delayed, it may result in decrease in *ε*-PL production. Various researchers reported similar trend of declining pH in *Streptomyces albulus* wherein decrease in pH is favorable for *ε*-PL production, but growth of *S. albulus* ceases at this pH and *ε*-PL production declines [31]. Therefore, such microrganism which can survive in such acidic pH can be better *ε*-PL producers. Hence, in our study, the halophilic bacteria *Bacillus licheniformis* is producing 80.1 mg/l *ε*-PL after 96 h which seems to be beneficial for commercial production as well. However, there is no report till date wherein any other strains of Bacillus licheniformis growing on pH 4.0 and producing *ε*-PL.

The effect of production age on *ε*-PL production by *Bacillus licheniformis* is shown in Figure 5. The fermentation medium adjusted to pH 8.5 prior to inoculation yielded 151 mg/l after 96 h. The bacteria start producing *ε*-PL extracellularly from 48 h and optimum production attains after 96 h having pH 4.0. This may be due to the attainment of the stationary phase after 96h. 155 mg/l of *ε*-Poly-L-lysine produced from *Bacillus licheniformis* is similar to 200 mg/l from a wild strain *Streptomyces albulus* CCRC 11814 reported in the literature [32,33].

The described present process may have a positive effect in reducing the product cost during the scale up at the bench scale and production scale. During fermentation processes at pilot scale, it is assumed that less production age will have a positive impact on the energy consumption, leading towards reduction in the product cost. Although, the raw material is having negligible cost as it is a waste residue of biodiesel process, less production age will add further impact in reducing the cost to a greater extent.

Furthermore, in the present process, two parameters which are helpful in its cost reduction are cheaper carbon source and less production age.
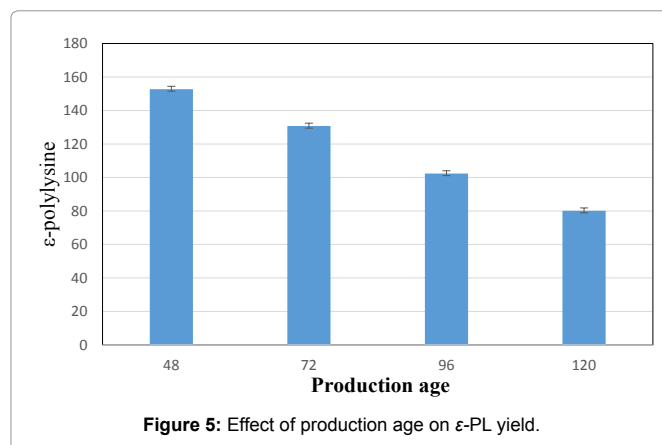
Downstream processing of *ε*-PL

Ammonium sulphate precipitation: The supernatant obtained after centrifugation was subjected to 40% ammonium sulphate saturation precipitated 7% of total protein present in the supernatant; 60% ammonium sulphate saturation precipitated 76.48% of total protein present in the supernatant; 80% ammonium sulphate saturation precipitated 38.77% of total protein present in the supernatant. Therefore, 60% ammonium sulphate saturation was considered further for precipitating *ε*-PL for recovery of maximum *ε*-PL.

Precipitation of polycationic *ε*-PL with TPB⁻ anion: 5 ml of 175 mM NaTPB solution in double distilled water added to 15 ml *ε*-PL concentrate, forming a cloudy white precipitate which was centrifuged out at 4°C, thereafter; precipitate was washed with distilled de-ionized water (DDI) water (5 ml × 3), digested in 10% aqueous hydrochloric acid, to obtain *ε*-PL hydrochloride which is insoluble in 10% hydrochloride solution [27]. The *ε*-PL hydrochloride was freeze dried under high vacuum.

It was reported that both NH_4^+ and K^+ forms precipitates with the TPB⁻ anion. Even after salting out for 12 h, some of the NH_4^+ and K^+ salts remained in the extract. However, it is difficult to remove all the NH_4^+ and K^+ during salting out process. During the additions of NaTPB to the extract having *ε*-PL, not only *ε*-PLHn⁺, but also NH_4^+, K^+, and monomeric lysine will be precipitated with the TPB⁻ anion as NH_4TPB and KTPB along with *ε*-PL-TPB.

Reaction of NaTPB with *ε*-PL

$$\varepsilon - PLH^{n+} + nNaTPB \rightarrow \varepsilon - PLH(TPB)_n \downarrow + nNa^+$$
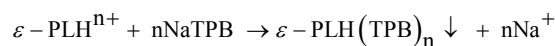
In order to purify *ε*-PL from the mixture of NH_4-TPB, K-TPB and *ε*-PL-TPB in aqueous phase, excess acetone was added for the dissolution of NH_4TPB and KTPB present in the leaving only *ε*-PL-TPB in the precipitate. NH_4TPB and KTPB will be soluble in some organic solvents and thus, excess acetone can be used for removing additional NH_4TPB and KTPB formed in the solution [28]. The obtained *ε*-PL-TPB after centrifugation was lyophilized to obtain the *ε*-PLTPB in powder form. Later, *ε*-PL hydrochloride was obtained from *ε*-PL-TPB through carrying the reaction with 10 % HCl and a total of 150 mg *ε*-PL hydrochloride was obtained.

Many researchers have purified *ε*-PL using conventional gel chromatography, Ion exchange chromatography, ultrafiltration, cross flow filtration, gel filtration chromatography, etc. However, purifying

ε-PL through chromatographic technique involves multistep purification and cost intensive, therefore, for a better sustainable process, the purification of ε-PL to be done in a single step through precipitating ε-PL by adding sodium tetraphenyl borate in the fermentation broth, which can obtained as ε-PL hydrochloride.

Characterization

SDS PAGE

The purified ε-PL hydrochloride was analyzed in SDS page. The left band in Figure 6 clearly depicts that the ε-PL is having 5.0 KDa molecular weight as the band of the standard ε-PL (5.0 KDa) is found on the same lane. The band of ε-PL was located at the similar position with respect to that of the standard one.

¹H NMR of ε-Polylysine in D₂O isolated from *Bacillus licheniformis*

Protons (Ha, Hc) attached to α-amino groups arrived together as broad singlet at δ 3.76 ppm and protons (Hb, Hd) attached to ε-amino groups arrived together as broad singlet at δ 3.14 ppm. Protons attached to β and β' carbons come at δ 1.75 ppm as broad singlet. While the other protons attached to carbons come at δ 1.47 ppm (4H, ε and ε') and δ 1.30 ppm (4H, γ and γ' respectively (Figure 6). Overall, the peaks showing peptide linkage between α-carboxyl group and the ε-amino group, confirming the structure as ε-polylysine (Figure 7).

Conclusion

Among obtained bacterial isolates from west coast of India, a

Figure 6: Poly acrylamide gel electrophoresis (PAGE) of *ε*-PL (left lane) against standard of *ε*-PL (right lane).
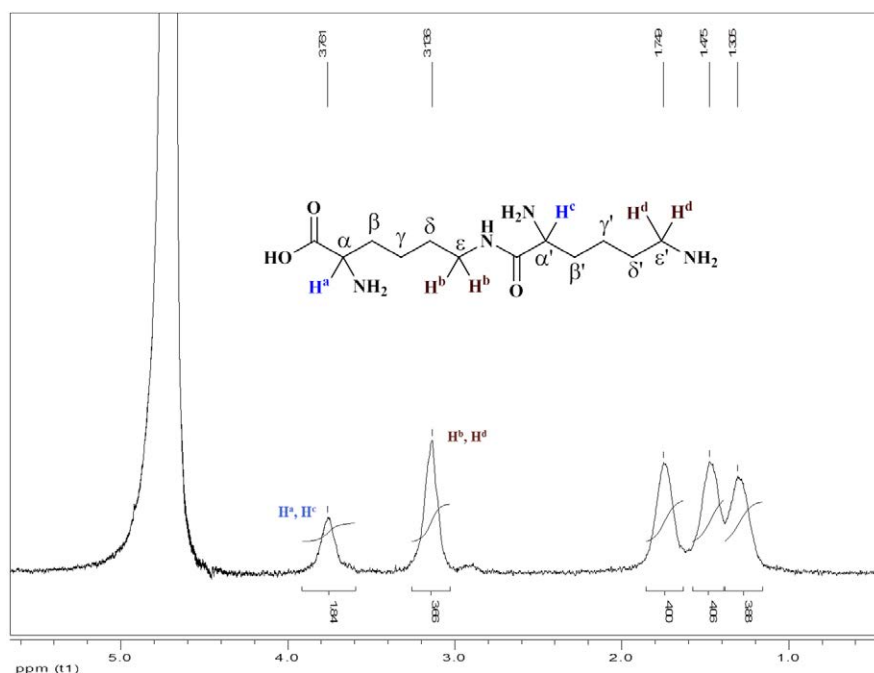

Figure 7: ¹H NMR of *ε*-PL in D₂O isolated from *Bacillus licheniformis*.

potential halophilic bacterial isolate identified as *Bacillus licheniformis* that can grow in crude glycerol and at the same time produces ε-PL at a concentration of 155 mg/l. However, other investigators have reported ε-PL production from *Streptomyces albulus* and *Bacillus sp.* with even higher yield (up to 565-2460 mg/l), but the study mentioned in literature is confined to pure glycerol/glucose as carbon source. Our study relates to utilization of crude glycerol (biodiesel waste residue) for the production of ε-PL from halophilic bacterial isolate *Bacillus licheniformis* isolated from brine of a salt pan. However, downstream processing of ε-PL was carried out through precipitation of ε-PL (present in the fermentation broth) with sodium tetraphenyl borate.

Acknowledgements

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