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Cloning, Expression and Genetic Regulation of a Biosurfactant Gene for Bioremediation of Hydrophobic Chemical Compounds

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The chromosomal DNA of *Bacillus subtilis* SK320 was amplified using *sfp0* gene (642bp) specific primers and the gene was cloned into *E. coli* DH5α using plasmid vector pGEM-T (3kb). Expression of the gene showed higher biosurfactant production (2.20 gm/lit) in the clone *E. coli* pSKP0, as compared to the parent *Bacillus subtilis* SK320 (1.2 gm/lit) when grown on olive oil as the sole substrate. Biosurfactant of Clone *E. coli* pSKP0 was found to be a powerful lipopeptide and reduced the surface tension of water from 72 to 35 dynes/cm as compared to 40.1 by *Bacillus subtilis* SK320. Expression studies during growth reveled the linkage of the biosurfactant to an esterase enzyme. Maximum biosurfactant activity was observed during the mid log phase of growth during which esterase activity was also maximum. Ion-exchange chromatography using Q Sepharose purified the extracellular esterase from pSKP0 and resolved it into three components; designated as P01, P02 and P03. All the three esterases were found to be heterogeneous in nature. Gel-filtration chromatography (Sephadex G-75) further resolved the esterases into their sub-components. The sub-components were further purified to homogeneity by poly-acrylamide gel electrophoresis as observed by activity and silver staining. The esterase enzyme from clone *E.coli* pSKP0 showed mol wt ranging from 14 to 120 kda and an isozyme pattern similar to *Bacillus subtilis* SK320.

Key words: Biosurfactants, olive oil, *Bacillus subtilis* SK320, esterase, isozyme, cloning, genetic regulation.

Biosurfactants or microbial surfactants are surface active biomolecules that are produced by a variety of microorganisms when grown on water immiscible or oily substrates. They remain adherent to microbial cell surfaces or are secreted in the culture broth. They possess the unique property of reducing the surface and interfacial tensions using the same mechanisms as chemical surfactants[1]. Biosurfactant production has steadily increased during the past decade owing to their wide variety of industrial and environmental applications. However large scale production of these molecules has not been realized because of

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low yields in production processes and high recovery and purification costs[2].

Low-molecular-mass biosurfactants such as glycolipids and lipopeptides generally act as detergents, lowering interfacial tension at liquid-liquid or liquid-solid interfaces[3,4]. Biodegradation of hydrocarbons is often associated with the production of surface-active compounds from microorganisms[5]. Emulsan, an amphipathic extracellular polyanionic bioemulsifier produced by *Acinetobacter venetianus* RAG-1 consists of D-galactosamine, L-galactosamine uronic acid (pKa, 3.05), and a diamino, 2-desoxy *n*-acetylglucosamine. In addition it also consists of a protein which contributes to its amphipathicity and to the hydrocarbon substrate specificity. The protein was found to be an esterase associated with the cell surface[6].

Surfactin is one of several microbially produced biosurfactants which are amphipathic molecules having many potential commercial applications[7]. Surfactin is synthesized in part by the multienzyme thiotemplate mechanism and is produced in the stationary phase cultures of *B. subtilis*[8]. The genes required for the biosynthesis of surfactin have been characterized to have three genetic loci i.e. *srfA* (an operon encoding at least some of the enzymes that catalyze surfactin synthesis), *srfB* (containing *comP* and *comA* genes which are required for competence development and transcription of *srfA*) and *sfp* (a gene of unknown function that is required for surfactin production)[9]. While the genes responsible for the biosynthesis and control of surfactin heteropolysaccharide have already been cloned and sequenced, little is known about the protein component(s) and the genetic regulation of the surfactin complex.

In this research work, we have cloned a biosurfactant gene *sfp0* from an endosulfan degrading *Bacillus subtilis* SK320 and over expressed it in *Escherichia coli* cells. We studied the genetic regulation of biosurfactant production and its correlation with the esterase activity. This observation is first to be reported in any *Bacillus* species. The industrial implications of this work are that the cloning was responsible for inducing the cells for mass production of biosurfactant which is an excellent lipopeptide effective in degrading/bioremediating a variety of hydrophobic chemical compounds. The recombinant *E. coli* cells containing the biosurfactant gene *sfp0* also showed esterase activity. Esterase was further purified to homogeneity and showed multiplicity by activity staining. An enhancement in the biosurfactant as well as esterase activity was observed in the clone *E.coli* pSKP0.

MATERIAL AND METHODS

Bacterial strains, vectors and medium used

Bacillus subtilis SK320 used in this study was isolated from endosulfan sprayed cashew plantation soil in Kerala, India. The isolate was identified as *Bacillus subtilis* by Microbial Type Culture Collection (MTCC), Chandigarh, India. *E. coli* strain DH5á was also obtained from MTCC. pGEM-T easy vector system was procured from Promega Corporation, Madison, USA and was used according to the manufacturers instruction. *Bacillus subtilis* SK320 will be addressed as *B subtilis* SK320 in the text.

B subtilis SK320 and clone *E. coli* pSKP0 used for enzyme purification were cultivated at 37°C, 120rpm in Bushnell Hass Broth (BHB) with 0.5% olive oil (v/v) (Olio di Oliva, Sasso, imported by Nestle India Ltd. from Milano, Italy) as carbon source. *E. coli* DH5á and *B subtilis* SK320 were maintained and sub cultured on luria agar (LA) plates and ampicillin (50µg/ml) was added to the medium with 0.5% olive oil (v/v) when *E. coli* DH5α cells harboring the recombinant plasmid were selected, respectively.

Cloning, expression and genetic regulation of the biosurfactant gene (sfp0)

Gene *sfp0* was PCR amplified using 5'-CTAGAATTCAGATTTACGGAATTTATATG-3' and 5'-GGGGAATTCAGGGTGTGCGGCG CATAC-3' primers[10] on GeneAmp PCR System 9700 (Applied Biosystems, Foster, CA, USA), using program set to denaturation at 94°C for 5 min, and then denature at 94°C for 1 min, anneal at 45°C for 1 min and extend at 72°C for 1 min for total of 30 cycles, with a final extension at 72°C for 10 min. The *sfp0* gene was ligated to pGEM-T easy vector system at 4^0C and cloned in *E. coli* DH5α [11]. The transformants were selected on Amp^+ X-gal IPTG plates. To check

expression the positive clones were grown in luria broth and basal medium containing 0.5% olive oil as carbon source, with ampicillin. Biosurfactant and esterase activities were estimated in the culture supernatant of the clone and the parent *B subtilis* SK320.

Partial purification of lipopeptide biosurfactant

For partial purification *B subtilis* SK320 was grown in BHB containing 0.5% olive oil (v/v) as carbon source. After 48hrs when the biosurfactant activity was observed to be maximum in the supernatant, the culture was harvested at 7,000 X *g* for 30 min at 4°C. Supernatant was filtered through Whatman No 42 filter paper and 3 volumes of chilled acetone was added to the supernatant and left at -20°C. After 18 hours the solution was centrifuged at 7000 *g* for 30 min at 4°C, allowed to be air dried and then re-dissolved in water. This step was repeated 2-3 times. The final precipitate was dissolved in water and the sample was lyophilized (Heto LyoLab 3000 Lyophilizer, Germany). The lyophilized sample was then estimated for biosurfactant activity at 550nm.

Assays

Esterase Activity

Esterase activity was measured using 100mM para-nitrophenyl (pNp) acetate as substrate and 75mM phosphate buffer containing 10mM $MgSO_4$ (pH 7.0). Enzyme activity was monitored spectrophotometrically by measuring the increase in optical density at 405 nm after 30 min of incubation at 37°c. Enzyme activity is expressed as mmoles/ml/min while specific activity is expressed as mmoles/mg protein/min[12].

Protein estimation

Protein in the culture broth was measured at 310 nm by Biuret method[13] using bovine serum albumin as standard. The fractions eluting from the column were analyzed for protein at 280nm.

Biosurfactant Activity

Biosurfactant activity was measured by using the culture supernatant directly. To the culture supernatant (5ml) taken in a glass tube obtained by centrifuging the bacterial growth at 12,000 X *g* at 4°C for 30 min[14], 100µl of mobile oil was added and the contents were vortexed vigorously for one minute at full speed and then left undisturbed for 10min. Biosurfactant or bioemulsifying (total emulsification obtained) activity was measured at 550nm spectrophotometrically (U-2001, Hitachi) in glass cuvette against blank of un-inoculated medium (5ml) with 100µl of mobile oil vortexed similar to the sample. The terms bioemulsifying activity and biosurfactant activity will be used interchangeably in this research paper.

Surface tension reduction (dynes/cm) was measured by ring method with the partially purified biosurfactant at a concentration of 1mg/ml in distilled water using a Du-Nouy tensiometer (local made) with distilled water as control.

Purification of esterase

Clone *E. coli* pSKP0 was grown on basal medium with 0.5% (v/v) olive oil and after 48hr old of growth, the culture was centrifuged at 10,000 X *g* for 30 minutes in a refrigerated high-speed centrifuge (Himach CR22G, Hitachi). Supernatant thus obtained was loaded onto a Q-sepharose column (43 × 3 cm) and eluted with the equilibrating 10mM potassium phosphate buffer (pH 7.0) at a flow rate of 30 ml hr^{-1} at 4°C. The bound proteins were eluted with a linear gradient of 1M NaCl in 10 mM phosphate buffer (pH 7.0). Protein and esterase activity were estimated spectrophotometrically (U-2001 spectrophotometer, Hitachi). Fractions showing maximum esterase activity were pooled, concentrated by lyophilization, dialyzed and then loaded onto a Sephadex G-75 column (72 X 2 cm) and fractions of 5ml were collected at a flow rate of 30ml/h. Active fractions were pooled and concentrated using amicon stirred ultra filtration assembly (Millipore) with a 10,000 Daltons cut-off, in cold (4°C) and then used for kinetic studies. Molecular weight of the purified esterase was determined by native PAGE analysis using mixture of standard molecular weight markers (MBI fermentas, USA).

Activity Staining

Purification of esterases was monitored by 10% continuous native polyacrylamide gel electrophoresis[15]. For activity staining for esterase, the gels after electrophoresis were washed with several changes of milli-Q water and then soaked in 100ml 0.2M phosphate buffer (pH 6.4) containing 100mg α-naphthyl acetate (alpha-NA), 100mg β-naphthyl acetate (beta-NA), 100mg Fast Blue RR salt (4-Benzoylamine-2, 5-dimethoxy benzene-diazonium chloride hemi

[zinc chloride] salt) in 3 ml acetone. Incubation was continued for 30 min at 37°C, in a gyratory shaker at a speed of 32 rpm [16].

RESULTS AND DISCUSSION

Cloning and expression of the biosurfactant gene (sfp0)

B subtilis SK320 chromosomal DNA was amplified by polymerase chain reaction using the *Bacillus* gene specific primers. The PCR product was ligated to the pGEM-T easy vector and transformed into *E. coli* DH5α. The positive clones obtained from the luria agar plates (containing ampicillin and X-gal IPTG) were selected and screened for biosurfactant activity. Out of the numerous colonies obtained clone *E. coli* pSKP0 was the most stable. The 3.7 kb plasmid from the recombinant *E. coli* pSKP0 cells on digestion with EcoRI resulted in 3000bp and 642bp fragments, respectively. The insert (642bp) was further restriction digested with RsaI (tetra-cutter) and HindIII (hexa-cutter). The cloned and sequenced gene showed 99% similarity with *Bacillus subtilis* surfactin synthetase subunit gene. The RFLP pattern and the BLAST results revealed that the *sfp0* gene was successfully cloned [DDBJ / EMBL / GeneBank Accession number, *sfp0* gene: EU822922].

B. subtilis SK320 utilized olive oil as carbon source for growth and showed growth dependent cell free biosurfactant activity and esterase activity. Expression studies reveled that the *E. coli* cells did not showed any growth in

Table 1. Purification of clone *E.coli* pSKP0 esterase

	Total Activity(IU)	Total Protein(mg)	Specific Activity (IU/mg)	% Recovery	Fold Purification
Supernatant Crude	4.92	176.63	0.03	100	0.00
Q Sepharose					
P01	0.25	4.70	0.05	4.54	1.79
P02	0.25	18.96	0.01	4.63	0.45
P03	0.49	24.80	0.02	9.02	0.67
Sephadex G 75					
P01a	0.17	5.54	0.03	3.15	1.05
P01b	0.17	3.85	0.04	3.17	1.52
P02a	0.52	3.73	0.14	9.56	4.74
P02b	0.49	6.74	0.07	8.98	2.46
P03a	0.66	5.18	0.13	12.04	4.30
P03b	0.50	7.04	0.07	9.16	2.40
P03c	0.18	6.14	0.03	3.32	1.00

Table 2. Kinetic properties of clone *E.coli* pSKP0 esterase

	Components						
	P01		P02		P03		
	P01a	P01b	P02a	P02b	P03a	P03b	P03c
Optimum pH	7	7	7	7	7	7	7
Optimum temp.	40	35	40	50	45	40	40
Km (µmoles)	4	3.90	2.97	1.23	2.93	1.63	2.21
Mw (KDa)	65	35	72	29	65	33	14
Vmax ($\mu molesmg^{-1}min^{-1}$)	0.412	0.112	0.439	0.529	0.127	0.433	0.312
KI (mM) $HgCl_3$	1.2	3.2	3.3	0.8	4.9	1.8	5.2
$PbCl_2$	2.5	3.3	2.0	0.5	2.3	1.5	1.7

basal medium containing 0.5% olive oil while clone *E. coli* pSKP0 when grown in basal medium containing 0.5% olive oil showed an increase in the growth. Extracellular biosurfactant activity in the culture broth as measured by the emulsification of mobile oil at 550nm increased with growth and was maximum at 48 hrs, showing that the cells produce maximum biosurfactant between 24 to 48 hrs. Cell free esterase activity also increased with growth with maximum activity of 4.92 U at 48 hrs. There was a sharp decline in the activity during the next 24hrs after which both the activities stabilized (Fig. 1).

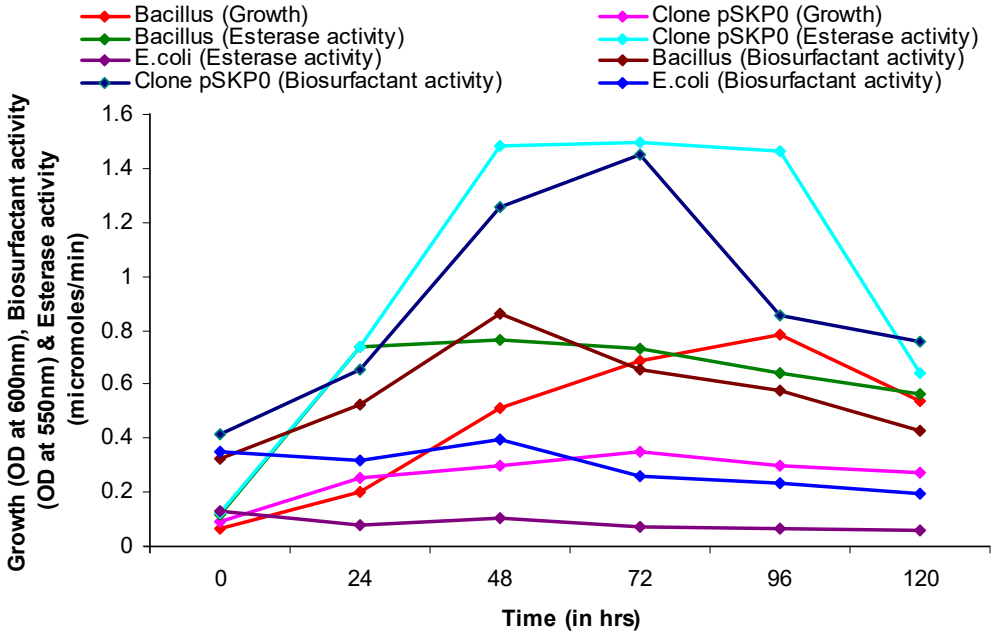

Fig 1. Growth, biosurfactant and esterase activity of *Bacillus subtilis* SK320, clone *E. coli* pSKP0 and *E.coli*, in basal medium containing 0.5 % (v/v) olive oil. Esterase activity has been multiplied with a factor of 10

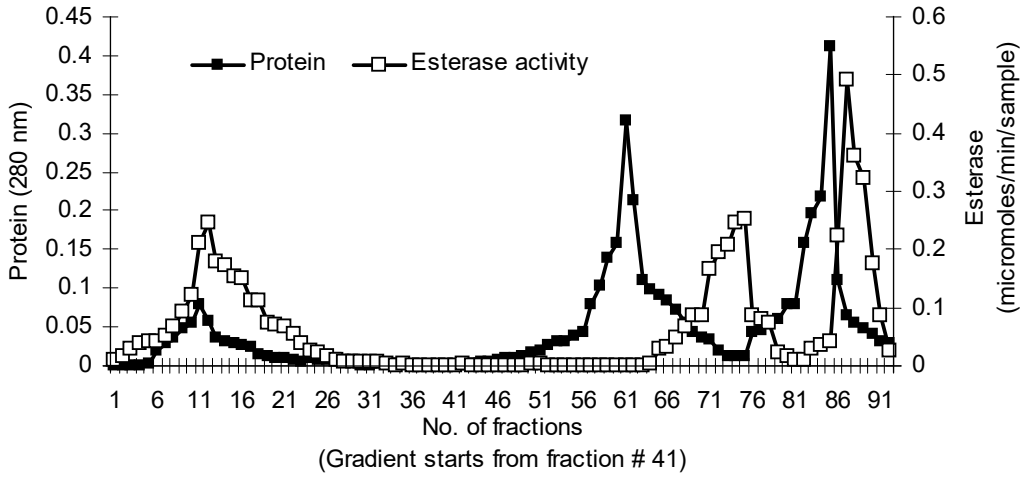

Fig. 2. Elution profile of esterase from clone *E. coli* pSKP0 on Q-Sepharose column

In *A calcoaceticus* BD 413 [17] high amount of esterase and biosurfactant activity was produced only during the transition from exponential to stationary phase, while in *A calcoaceticus* RAG-1 [6] esterase activity followed the growth pattern, with the maximum activity being achieved during the stationary phase of growth. Similar increase in lipase, an ester hydrolase activity during transition to stationary phase has been reported in *A calcoaceticus*[17]. In *Acinetobacter venetianus* RAG-1 the release of emulsan from the bacterial cell surface was mediated by the action of a cell surface esterase, which is one of the key components in the active emulsan-protein complex and itself appears in the growth medium just prior to the appearance of the cell-free emulsifying activity[6]. The mutants of RAG-1 defective in esterase were found to be defective in emulsan production and release[18].

Biochemical analysis revealed that the partially purified biosurfactant from clone *E. coli* pSKP0 was a lipoprotein with a high lipid (90.3%) content and 6.73 % protein with a very low carbohydrate content of 2.94%. The ash content was 15.2 %. The biosurfactant from *B subtilis* SK320 was also a lipoprotein with similar biochemical characteristics. The biosurfactant from clone *E. coli* pSKP0 was able to reduce the surface tension of water from 72 to 35 dynes/cm, whereas the biosurfactant from the parent *B subtilis* SK320 reduced the surface tension to 40.1 dynes/cm. Clone *E. coli* pSKP0 when grown on olive oil showed an enhancement in biosurfactant production due to the over-expression of *sfp0* gene

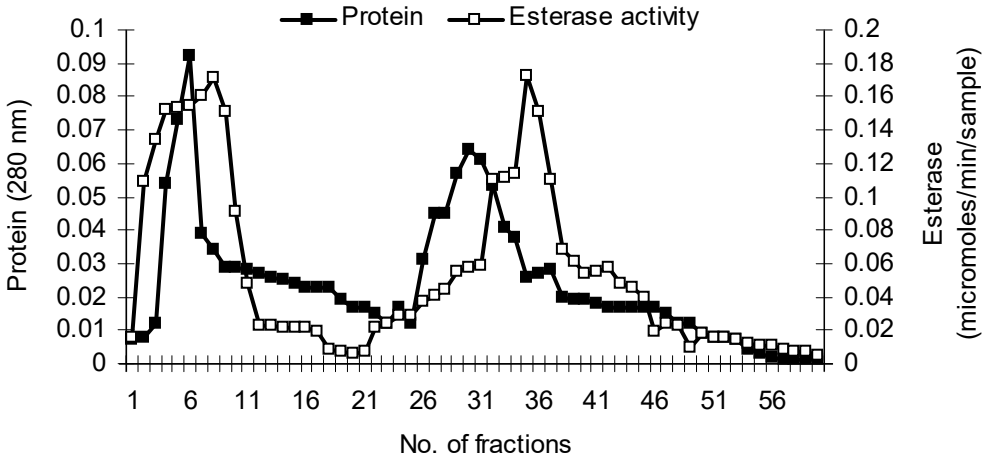

Fig. 3. Elution profile of esterase component P01 on Sephadex G-75 column.

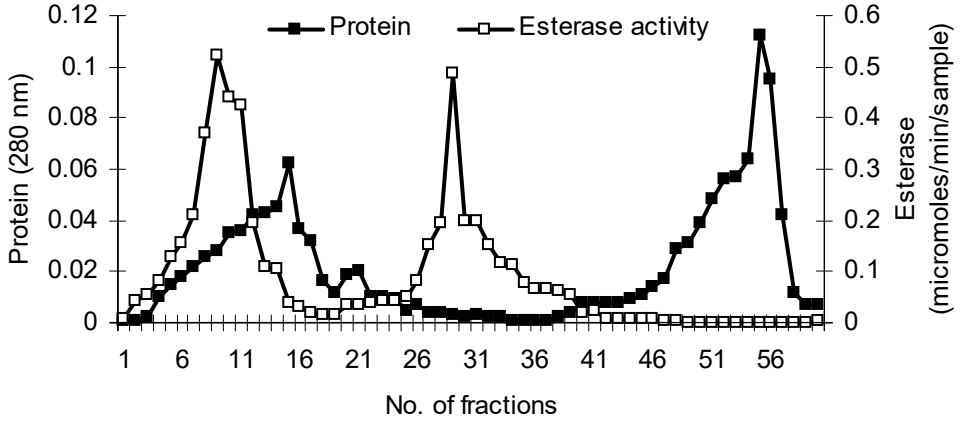

Fig. 4. Elution profile of esterase component P02 on Sephadex G-75 column.

in the clone. The production yield of partially purified biosurfactant from clone *E. coli* pSKP0 was 2.20 gm/lit, which was almost double to the production (1.2 gm/lit) observed in the parent *B subtilis* SK320. In halotolerant *Bacillus subtilis* BBK-1, yield of 480 mg/lit at 30°C was observed from 24hrs culture supernatant [10] whereas 1 gm/lit was the biosurfactant yield obtained from the two other *Bacillus* species[14].

Formation of emulsion in presence of olive oil by the recombinant *E. coli* cells, and observing the high biosurfactant activity suggests that the biosurfactant produced by the clone is capable of hydrolyzing various hydrophobic chemical compounds. In about 48 to 72 hrs of growth the clone *E. coli* pSKP0 was able to completely emulsify/dissolve/degrade the olive oil present in the media suggesting that the biosurfactant produced by the clone *E. coli* pSKA acts as an excellent emulsifier or surface active molecule which could help in enhancing the bioremediation process. This is also supported by the observation that the biosurfactant produced by clone *E. coli* pSKP0 significantly lowers the surface tension of water from 72 to 35 dynes/cm, when a concentration of as low as 10 mg/lit was used, indicating that the biosurfactant has a very low critical micelle concentration (CMC). The CMC of clone *E. coli* pSKP0 is two fold lower then surfactin (25 mg/lit) [19]. Purified lichenysin A produced by *B. licheniformis* BAS50 decreases the surface tension of water to 28mN/m and achieves the CMC of 12 mg/lit, with the production yield of 160mg/lit [20]. The lowest interfacial tension ever reported for a microbial surfactant was 6×10^{-3} dyne/cm with a CMC of 10mg/lit by the biosurfactant of *B. licheniformis* JF-2[21].

Purification of* clone *E. coli* pSKP0 *esterase

Esterase from clone *E. coli* pSKP0 was purified from the supernatant (4.92 IU) of the mid log phase cells grown in basal medium amended with 0.5% olive oil as the sole carbon source. Under these conditions esterase accumulates in the medium with no apparent loss of activity. The extracellular protein obtained from the clone *E. coli* pSKP0 was purified by Q-sepharose followed by Sephadex G-75. Ion-exchange chromatography on Q-sepharose resolved the supernatant into three distinct active protein components (Fig. 2) giving maximum esterase activity. The protein eluting in the equilibrating buffer (P01, cationic) constituted only 9.6% of the total esterase activity. Fractions (P01) pooled from Q Sepharose were concentrated by ultrafiltration and loaded onto Sephadex G 75. Two activity peaks were obtained and designated as P01a and P01b. Esterase P01a and P01b with sp act 3.15 IU and 3.17 IU (Table 1) were purified 1.05 and 1.52 % respectively (Fig. 3).

The second protein component (P02, anionic) on Q Sepharose was eluted at 700mM NaCl using a 10 – 1000mM NaCl gradient in

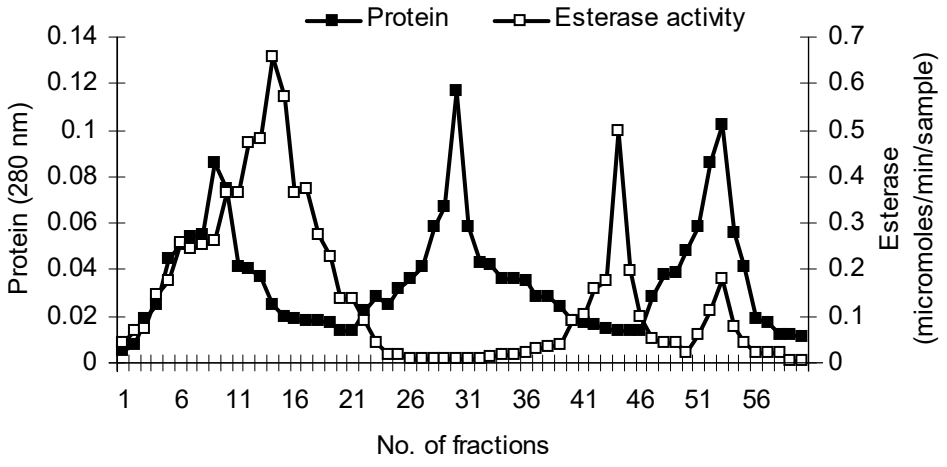

Fig. 5. Elution profile of esterase component P03 on Sephadex G-75 column

10mM sodium phosphate buffer (pH 7.0) and constituted 39% of the total activity. Active fractions were pooled, dialyzed, concentrated by ultrafiltration and then loaded onto Sephadex G 75. Component P02 was resolved into two components with esterase activity (Fig. 4). These were designated as esterase P02a and P02b respectively and had sp act of 9.56 and 8.98 IU (Table 1).

The third active protein component (P03, anionic) on Q Sepharose eluted at 940mM NaCl and on Sephadex G 75 was resolved into three active protein components: P03a, P03b and P03c (Fig. 5) with sp act of 12.04, 9.16 and 3.32 IU, respectively (Table 1).

The elution profile of ion exchange (Fig. 2) and gel-filtration (Fig. 3, 4, 5) chromatography of clone *E. coli* pSKP0 was found to be similar to the parent strain (data not shown).

Esterase enzyme multiplicity in the supernatant could be due to some post-translational modification or some different gene products encoded by the bacterial genome. Multiplicity of esterases has been reported in *Bacillus* species particularly in *B. coagulans*, *B. subtilis* & *B. acidocaldarins, in Pseudomonas fluorescens*[22-27] and in *T. reesei*[28] and *P. purpurogenum*[29]. The role of multiplicity can only be explained that despite esterases not being essential for growth in microbes, the multiplicity allows them to function for the hydrolysis of ester compounds for their better assimilation.

The kinetic properties of purified esterases are shown in Table 2. The enzyme shows strong activity in the pH range of 6-8 with optimum pH at 7. All components of esterase were stable at 40°C except the P01b, P02b and P03a components which were stable at 35, 50 and 45°C, respectively. Michaelis (Km) constant for P01a, P01b, P02a, P02b, P03a, P03b and P03c was calculated as 4, 3.90, 2.97, 1.23, 2.93, 1.63, 2.21 µmoles whereas the Vmax values were found to be 0.412, 0.112, 0.439, 0.529, 0.127, 0.433, 0.312 µmol min^{-1} mg^{-1}, respectively. In *B. subtilis* the cephalosporin esterase exhibited Km values of 2.8×10^{-3} and 8.3×10^{-3} M, for substrates 7-aminocephalosporanic acid (7-ACA) and 7-(thiophene-2-acetamido) cephalosporanic acid (cephalothin) [30]. Km and Vmax of 0.88 µmoles and 8.9 U/mg for 6-acetylmorphine was found for the purified heroin esterase from *E. coli* clone of *Rhodococcus* sp. strain H1[31], Km values of 0.45 and 0.52 µmoles were observed for acetyl xylan esterases I and II from *Thermoanaerobacterium* sp. strain JW/SL-YS485, when 4-methylum belliferyl acetate was used as the substrate[32] whereas for the esterase from *R. toruloides* the Km and Vmax were found to be 51.8 µmoles and 7.9 µmol min^{-1} mg^{-1} respectively, when cephalosporin C was used as a substrate[12]. Esterases components were strongly inhibited by Hg, Pb and EDTA with KI (mM) of 1.2, 3.2, 3.3, 0.8, 4.9, 1.8 and 5.2 for components P01a, P01b, P02a, P02b, P03a, P03b and P03c with $HgCl_3$, whereas with $PbCl_2$ as inhibitor the values observed were 2.5, 3.3, 2.0, 0.5, 2.3, 1.5 and 1.7, respectively.

The Mw (kDa) of the esterase components were 65, 35, 72, 29, 65, 33 and 14 for P01a, P01b, P02a, P02b, P03a, P03b and P03c, respectively (Fig. 6). The enzymes exits as a monomer and this is in contrast to that reported

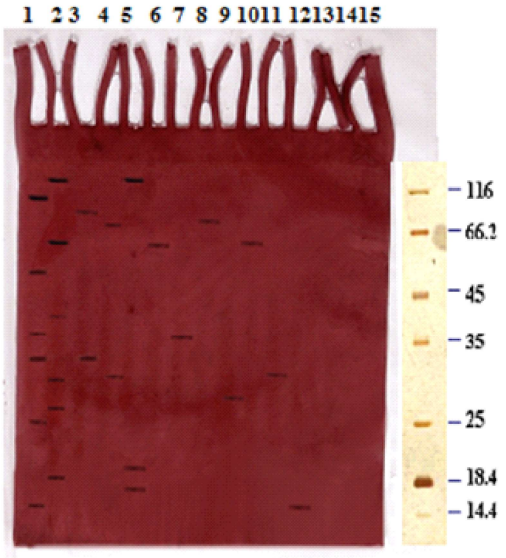

Fig. 6. Native PAGE analyses of esterase from clone *E. coli* pSKP0. The protein at different steps of purification was analyzed by 10% native PAGE followed by activity staining by fast blue RR salt. Lane 1: *Bacillus subtilis* SK320 supernatant, Lane 2: clone *E. coli* pSKP0 supernatant, Lane 3, 4, 5: Q-sepharose P01, P02, P03, Lane 6 to 12: G-75 P01a, P01b, P02a, P02b, P03a, P03b, P03c

in a study[33] where the *Bacillus subtilis* enzymes are multimeric in nature with the molecular weights ranging from 150 to 280 kDa.

CONCLUSION

sfp0 gene was successfully cloned and expressed in *E.coli* and showed enhancement in biosurfactant production. The biosurfactant production showed correlation with esterase activity. The biosurfactant produced by *Bacillus subtilis* SK320 was a lipopeptide with strong emulsifying properties thus has wide range of industrial and agricultural applications in bioremediation of the hydrophobic chemical compounds ranging from sites contaminated with poly aromatic hydrocarbons to polychlorinated biphenyls to emulsifying the immiscible organophosphorus pesticides. Esterases coded by *sfp0* gene of *Bacillus subtilis* SK320 were purified, characterized and found to exist as allozymes. This is the first report of a biosurfactant showing esterase enzyme induction in any *Bacillus* species.

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