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Cloning and Expression of a Biosurfactant Gene from Endosulfan Degrading *Bacillus* sp.: Correlation Between Esterase Activity and Biosurfactant Production

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Abstract: The *srfA* gene (1976 bp) was cloned from *Bacillus* sp. SK320 into *E. coli* DH5α using plasmid vector pGEM-T (3 kb). Higher esterase activity was observed in the clone *E. coli* pSKA with olive oil as sole carbon source, as compared to that from *Bacillus* sp. SK320. Purification of esterase from *E. coli* (pSKA) on Q-Sepharose resolved the extracellular esterase into three components designated as A1, A2 and A3. All the three esterases were heterogeneous in nature. Sephadex G-75 further resolved the esterase into sub components which were purified to homogeneity as seen by activity as well as silver staining. Clone *E. coli* pSKA showed esterase enzyme with mol. wt. ranging from 12 to 53 Da indicating the multiplicity of the enzyme. An extracellular esterase from clone *E. coli* pSKA grown on olive oil was purified and shown to possess biosurfactant activity. Clone *E. coli* pSKA showed an enhancement in the biosurfactant production (2.45 g L^{-1}) as compared to 1.2 g L^{-1} from *Bacillus* sp. SK320. Clone *E. coli* pSKA reduced the surface tension to 32 dynes cm^{-1} as compared to 40 dynes cm^{-1} by *Bacillus* sp. SK320.

Key words: Biosurfactants, olive oil, *Bacillus* sp., esterase, cloning

INTRODUCTION

Biosurfactants have been used in a variety of industrial and environmental applications. Low-molecular-mass biosurfactants such as glycolipids and lipopeptides generally act as detergents, lowering interfacial tension at liquid-liquid or liquid-solid interfaces (Arima *et al*., 1968; Desai *et al*., 1994).

Biodegradation of hydrocarbons is often associated with the production of surface-active compounds from microorganisms (Koch *et al*., 1991). 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 (Gutnick *et al*., 2003).

Surfactin is one of several microbially produced biosurfactants which are amphipathic molecules having many potential commercial applications (D'Souza *et al*., 1994). Surfactin is synthesized in part by the multienzyme thiotemplate mechanism and is produced in the stationary phase cultures of *B. subtilis* (Kluge *et al*., 1988). 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) (Nakano *et al*., 1992). While, the genes responsible for the biosynthesis and control of surfactin heteropolysaccharide have recently been cloned and sequenced, little is known about the protein component(s) of the surfactin complex.

We have cloned a biosurfactant gene *srfA* from an endosulfan degrading *Bacillus* sp. SK320 and over expressed it in *Escherichia coli* cells. *srf*A (1976 bp) gene loci is present in *Bacillus* species only and its product includes lipopeptide biosurfactant, surfactin and several other lipoproteins. The recombinant *E. coli* cells containing the biosurfactant gene *srfA* showed esterase activity, which was purified to homogeneity and which showed multiplicity by activity staining. An enhancement in the esterase as well as biosurfactant activity was observed in the clone *E. coli* pSKA. We also observed

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that there is some potential correlation between biosurfactant production and esterase activity in the clone *E. coli* pSKA. This observation is the first to be reported in any *Bacillus* species.

MATERIALS AND METHODS

Bacterial strains and vectors: *Bacillus* sp. SK320 used in this study was isolated from endosulfan sprayed cashew plantation soil in Kerala (India) in 2003 and identified as *Bacillus* sp. by Microbial Type Culture Collection (MTCC), Chandigarh, India. *E. coli* DH5α was also obtained from MTCC. pGEM-T easy vector system was obtained from Promega Corporation, Madison, USA and was used according to the manufacturers instructions.

Bacillus sp. SK320 and clone *E. coli* pSKA used for enzyme purification were cultivated at 37°C, 120 rpm 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 and also as an inducer for the production of the biosurfactant. *Bacillus subtilis* SK320 grown LA did not produce biosurfactant. *Escherichia coli* DH5α and *Bacillus* sp. SK320 were maintained and sub cultured on Luria Agar (LA) plates and ampicillin (50 μg mL^{-1}) was added to the medium with 0.5% olive oil when *E. coli* DH5α harboring the recombinant plasmid were selected, respectively.

Cloning, transformation and expression of the biosurfactant gene (*srfA*): *Bacillus* sp. SK320 chromosomal DNA was extracted using a method by Rose *et al*. (1994). PCR amplification of *Bacillus subtilis* SK320 chromosomal DNA was carried out and PCR product was used for ligation. *srfA* gene was PCR amplified using 5'-TCCGTTTTTCCTTGTTCACC-3' and 5'-TCTTTCTGCCACTGCATCAC-3' self-designed primers 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 primers were prepared by Operon Biotechnologies, Nattermannallee, Germany (www.operon.com). The *srfA* gene was ligated to pGEM-T easy vector system at 4°C and transformed in *E. coli* DH5α (Cohen *et al*., 1972). The transformants were selected on Amp^{+} X-gal IPTG plates. The nucleotide sequence of the insert was determined by the dideoxy-chain termination method (Sanger *et al*., 1977) using Applied Biosystems DNA sequencer.

Expression studies were carried out by growing the positive clones in luria broth as well as basal medium containing 0.5% olive oil as carbon source, with ampicillin. Esterase and biosurfactant activities were estimated in the culture supernatant of the clone as well as the parent *Bacillus* sp. SK320.

Assays: Esterase was measured by using 100 mM para-nitrophenyl (pNp) acetate as substrate and 75 mM phosphate buffer containing 10 mM $MgSO_4$ (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. Specific activity is expressed as μmol/mg protein/min (Politino *et al*., 1997).

Protein in the supernatant was measured at 310 nm by Biuret method (Itzhaki and Gill, 1964) using bovine serum albumin as standard. The fractions eluting from the column were analyzed for protein at 280 nm.

Bioemulsifying activity of the biosurfactant was measured with the culture supernatant obtained by centrifuging the bacterial growth at 12,000 x g at 4°C for 30 min (Desai and Banat, 1997). To 5 mL of supernatant in a glass tube, 100 μL of mobile oil was added and the contents were vortexed vigorously for one minute at full speed and then left undisturbed for 10 min. Bioemulsifying activity was measured at 550 nm spectrophotometrically (U-2001, Hitachi) in glass cuvette against blank of un-inoculated medium (5 mL) with 100 μL of mobile oil vortexed similar to the sample. Surface tension reduction was measured with the partially purified biosurfactant at a concentration of 1 mg mL^{-1} in distilled water using a tensiometer with distilled water as control.

Purification of esterase: A 48 h old culture of clone *E. coli* pSKA grown on basal medium with 0.5% (v/v) olive oil was centrifuged at 10,000 X g for 30 min in a refrigerated high-speed centrifuge (Himach CR22G, Hitachi). Supernatant (S_{10}) was loaded onto a Q-Sepharose column (43×3 cm) and first eluted with the equilibrating 10 mM potassium phosphate buffer (pH 7.0) at a flow rate of 30 mL h^{-1} at 4°C and the bound protein were eluted with a linear gradient of 1 M NaCl in 10 mM phosphate buffer (pH 7.0). Protein and esterase activity were estimated spectrophotometrically (U-2001 spectrophotometer, Hitachi).

Molecular weight of the esterase was determined by native PAGE analysis using mixture of molecular weight markers.

Fractions showing esterase activity were pooled, concentrated by lyophilization and then dialyzed. Enzyme preparation (3 mL) was loaded onto a Sephadex G-75 column (72×2 cm) and fractions of 5 mL were collected at a flow rate of 30 mL h^{-1}. Active fractions were pooled and concentrated using amicon stirred ultra filtration assembly (Millipore) with a 10,000 Daltons cut-off, in cold (4°C).

Dialysis (Maniatis *et al.*, 1982) of the fractions involved retention of proteins of ≥12,000 kDa in the dialysis tubing. These fractions were then used for kinetic studies.

Activity staining: Purification of esterases was monitored by 10% continuous native polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). For activity staining for esterases the gels after electrophoresis were washed with several changes of milli-Q water and then soaked in 100 mL 0.2 M phosphate buffer (pH 6.4) containing 100 mg α-naphthyl acetate (alpha-NA), 100 mg α-naphthyl acetate (beta-NA), 100 mg Fast Blue RR salt (4-Benzoylamine-2, 5-dimethoxy benzene-diazonium chloride hemi [zinc chloride] salt) or Fast Blue B salt in 3 mL acetone. For RR salt, incubation was continued for 30 min, whereas in case of B salt the incubation period was 20 min at 37°C, respectively in a gyratory shaker at a speed of 32 rpm (Liu *et al.*, 1992).

RESULTS AND DISCUSSION

Cloning of biosurfactant gene (*srfA*): Chromosomal DNA of *Bacillus* sp. SK320 was amplified by polymerase chain reaction using the self designed *Bacillus* gene specific primers. The PCR product was ligated to the pGEM-T easy vector and transformed into *E. coli* DH5α. The colonies on luria agar plates containing ampicillin and X-gal IPTG were screened for biosurfactant activity. The clone *E. coli* pSKA was the most stable and selected for further studies. The 3.7 kb plasmid from the recombinant *E. coli* pSKA cells on digestion with EcoRI resulted in a 3000 and 727 bp fragments, respectively. The nucleotide sequence of the product as determined by the dideoxy-chain termination method was submitted to NCBI (Pub Med Accession No. EU822923).

Clone *E. coli* pSKA which was grown in basal medium containing 0.5% olive oil showed a gradual increase in growth and along with growth extracellular biosurfactant activity also increased reaching a peak at 48 h. *Bacillus subtilis* SK320 grown only on LA did not produce biosurfactant, whereas when grown on olive oil it produced substantial amount of biosurfactant. We had grown and checked biosurfactant production on different carbon sources (data not shown) as well, but olive oil was found to be the best inducer for biosurfactant production. Cell free esterase activity also increased with growth with maximum activity of 0.8 U at 48 h. Both cell free biosurfactant activity and cell free esterase activity declined steadily during the stationary phase of growth. *Bacillus* sp. SK 320 utilized olive oil as carbon source for growth and showed growth dependent cell free biosurfactant activity and cell free esterase activity,

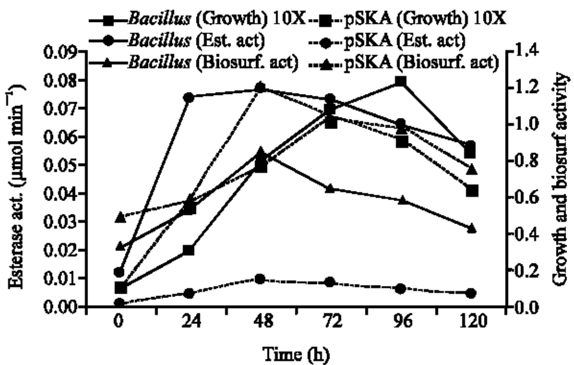

Fig. 1: Esterase activity of *Bacillus* sp. SK320 and clone *E. coli* (pSKA) in BHB containing 0.5% (v/v) olive oil

although both cell free biosurfactant activity and cell free esterase activity were maximum at 48 h at mid log phase of growth. There was a sharp decline in the activity during the next 24 h after which both the activities stabilized (Fig. 1).

In *A. calcoaceticus* BD 413 (Kok *et al.*, 1993) high amount of esterase and biosurfactant activity was produced only during the transition from exponential to stationary phase, while in *A. calcoaceticus* RAG-1 (Gutnick *et al.*, 2003) 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* (Kok *et al.*, 1993). 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 (Gutnick *et al.*, 2003). The mutants of RAG-1 defective in esterase were found to be defective in emulsan production and release (Shabtai and Gutnick, 1985).

Biochemical analysis revealed that the partially purified biosurfactant from clone *E. coli* pSKA was a lipoprotein with a high lipid (91%) content and 6.26% protein. The ash content was 15.38% whereas the biosurfactant had a very low carbohydrate content of 2.70% (data not shown). The biosurfactant from *Bacillus* sp. SK320 was also a lipoprotein with similar biochemical characteristics but the biosurfactant from clone *E. coli* (pSKA) was able to reduce the surface tension of water from 72 to 30.7 dynes cm^{-1}, whereas the biosurfactant from *Bacillus* sp. SK320 had a potential to reduce the surface tension to 40.1 dynes cm^{-1}. *Escherichia coli* (pSKA) when grown on olive oil

Table 1: Purification of clone *E. coli* (pSKA) esterase

Variables	Total activity (IU)	Total protein (mg)	Specific activity (IU mg^{-1})	Fold purification
Crude	172.0	134.00	1.28	0.00
Q Sepharose				
A1	7.60	10.60	0.72	0.56
A2	8.30	49.50	0.17	0.13
A3	15.60	63.07	0.25	0.19
Sephadex G 75				
A1a	10.35	1.23	8.41	6.56
A1b	9.85	1.01	9.75	7.60
A2a	23.35	1.12	20.85	16.24
A2b	28.50	4.12	6.92	5.39
A3a	35.52	1.15	30.89	24.06
A3b	24.89	2.11	11.80	9.19
A3c	11.30	2.56	4.41	3.44

showed an enhancement in biosurfactant production due to the over-expression of *srfA* gene in the clone. The production yield of partially purified biosurfactant from clone *E. coli* (pSKA) was 2.45 g L^{-1}, which was almost double to the production (1.2 g L^{-1}) observed in the parent *Bacillus* sp. SK 320. In halotolerant *Bacillus subtilis* BBK-1 yield of 480 mg L^{-1} at 30°C was observed from 24 h culture supernatant of (Roongsawang *et al*., 2002) whereas 1 g L^{-1} was the biosurfactant yield obtained from the two other *Bacillus* species (Cooper and Goldenberg, 1987).

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 hydrocarbons. In about 48 to 72 h of growth the clone *E. coli* pSKA was able to completely emulsify the olive oil present in the media suggesting that the biosurfactant produced by the clone *E. coli* pSKA acts as a good emulsifier. This is also supported by the observation that the biosurfactant produced by clone *E. coli* pSKA significantly lowers the surface tension of water from 72 to 30.7 dynes cm^{-1} (Table 1), that also when a concentration of as low as 10 mg L^{-1} was used, indicating that the biosurfactant has a very low Critical Micelle Concentration (CMC). The CMC of clone *E. coli* pSKA is two fold lower then surfactin (25 mg L^{-1}) (Cooper *et al*., 1981). Purified lichenysin A produced by *B. licheniformis* BAS50 decreases the surface tension of water to 28 mN m^{-1} and achieves the CMC of 12 mg L^{-1}, with the production yield of 160 mg L^{-1} (Yakimov *et al*., 1995). The lowest interfacial tension ever reported for a microbial surfactant was 6×10^{-3} dyne cm^{-1} with a CMC of 10 mg L^{-1} by the biosurfactant of *B. licheniformis* JF-2 (Lin *et al*., 1994).

Purification of esterase from clone *E. coli* (pSKA): We had conducted qualitative plate test (Falcocehio *et al*., 2005) to demonstrate that this enzyme is an esterase. Esterase activity was observed by direct plate test based

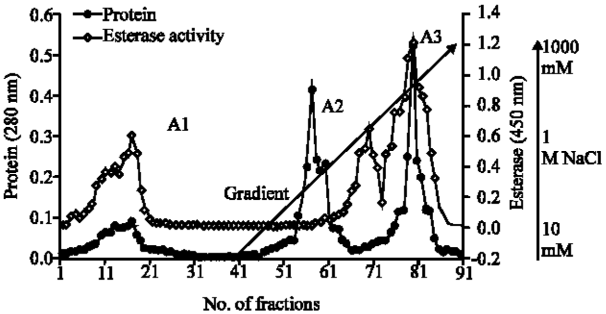

Fig. 2: Elution profile of esterase from clone *E. coli* (pSKA) on Q-Sepharose column

on streaking the isolated strain on agar plates supplemented with 0.5% (v/v) olive oil. After 48 to 72 h incubation at 37°C the appearance of hydrolysis haloes visible around the white glistening colonies indicated esterase activity.

Esterase from clone *E. coli* pSKA was purified from the supernatant 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* (pSKA) 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 (A1, cationic), constituted only 8% of the total esterase activity. Fractions (A1) pooled from Q-Sepharose were concentrated by ultrafiltration and loaded onto Sephadex G 75. Two activity peaks were obtained and designated as A1a and A1b. Esterase A1a and A1b (sp act 8.4 and 9.75 IU) were purified 6.5 and 7.6%, respectively (Fig. 3).

The second protein component (A2, anionic) on Q-Sepharose was eluted at 620 mM NaCl using a 10-1000 mM NaCl gradient in 10 mM sodium phosphate buffer (pH 7.0) and constituted 5% of the total activity. Active fractions were pooled, dialyzed, concentrated by ultrafiltration and then loaded onto Sephadex G 75.Component A2 was resolved into two components with esterase activity (Fig. 4). These were designated as esterase A2a and A2b, respectively and had sp act of 20.85 and 6.92 IU (Table 1).

The third active protein component (A3, anionic) on Q Sepharose eluted at 820 mM NaCl and on Sephadex G 75 was resolved into three active protein components (A3a, A3b and A3c) with sp act of 30.89, 11.80 and 4.41 IU for A3a, A3b and A3c, respectively (Fig. 5). The elution profile of ion exchange (Fig. 2) and gel-filtration (Fig. 3-5) chromatography of clone *E. coli* pSKA was found to be

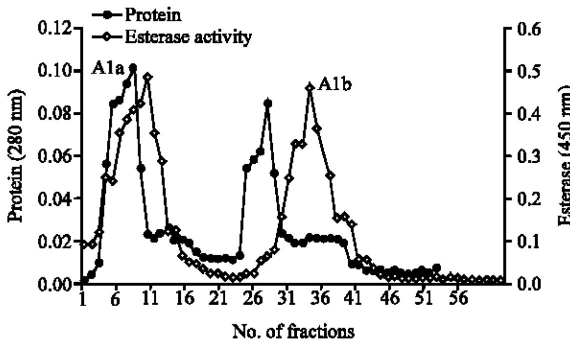

Fig. 3: Elution profile of esterase component A1 on Sephadex G-75 column

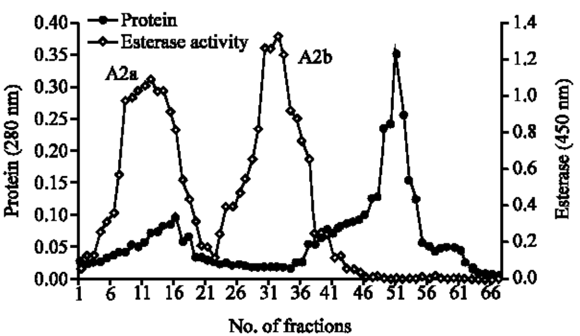

Fig. 4: Elution profile of esterase component A2 on Sephadex G-75 column

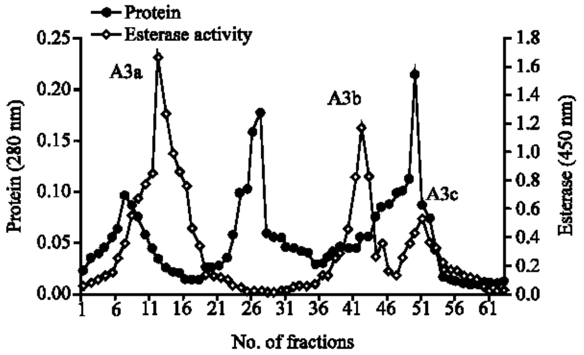

Fig. 5: Elution profile of esterase component A3 on Sephadex G-75 column

similar to the parent strain (data not shown) i.e., the presence of three esterases P1 (cationic), P2 and P3 (anionic) in the culture supernatant. 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* sp. particularly in *B. coagulans*, *B. subtilis* and *B. acidocaldarins*, in *Pseudomonas fluorescens*

Table 2: Kinetic properties of the clone *E. coli* (pSKA) esterase

	A1		A2		A3		
Properties	A1a	A1b	A2a	A2b	A3a	A3b	A3c
Optimum pH	7.00	7.00	7.00	7.00	7.00	7.00	7.00
Optimum temp.	40.00	40.00	35.00	55.00	50.00	40.00	45.00
Km (μmol)	2.38	1.67	3.06	2.70	2.80	1.60	1.98
Mw (kDa)	53.00	45.00	53.00	33.00	55.00	16.00	12.00
V_{max} (μmol/mg/min)	0.168	0.332	0.612	0.167	0.413	0.520	0.608
KI (mM)							
$HgCl_3$	0.55	2.80	3.20	0.80	3.30	1.90	4.50
$PbCl_2$	1.20	1.90	5.50	0.20	4.10	2.10	1.30

(Mnisi *et al*., 2005; Higerd and Spizizen, 1973; Manco *et al*., 1994, 2000; Choi *et al*., 1990; Khalameyzer *et al*., 1999) and in *T. reesei* (Biely *et al*., 1987) and *P. purpurogenum* (Egana *et al*., 1996). 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 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 A3a and A2b component which were stable also at 50 and 55°C, respectively. Michaelis (Km) constant for A1a, A1b, A2a, A2b, A3a, A3b and A3c was calculated as 2.38, 1.67, 3.06, 2.7, 2.8, 1.60, 1.98 μmol whereas the V_{max} values were found to be 0.168, 0.332, 0.612, 0.167, 0.413, 0.520, 0.608 μmol/min/mg, 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) (Abbott and Fukuda, 1975). Km and V_{max} of 0.88 μmol and 8.9 U mg^{-1} for 6-acetylmorphine was found for the purified heroin esterase from *E. coli* clone of *Rhodococcus* sp. strain H1 (Rathbone *et al*., 1997), Km values of 0.45 and 0.52 μmol were observed for acetyl xylan esterases I and II from *Thermoanaerobacterium* sp. strain JW/SL-YS485, when 4-methylumbelliferyl acetate was used as the substrate (Shao and Wiegel, 1995) whereas for the esterase from *R. toruloides* the Km and V_{max} were found to be 51.8 μmol and 7.9 μmol/min/mg, respectively, when cephalosporin C was used as a substrate (Politino *et al*., 1997). Esterases components were strongly inhibited by Hg, Pb and EDTA with KI (mM) of 0.55, 2.8, 3.2, 0.8, 3.3, 1.9 and 4.5 for components A1a, A1b, A2a, A2b, A3a, A3b and A3c with $HgCl_3$, whereas with $PbCl_2$ as inhibitor the values observed were 1.2, 1.9, 5.5, 0.2, 4.1, 2.1 and 1.3, respectively.

The M. wt. (kDa) of the esterase components were 53, 45, 53, 33, 55, 16 and 12 for A1a, A1b, A2a, A2b, A3a, A3b

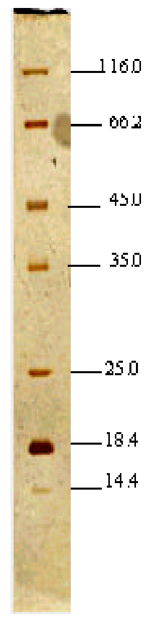

Fig. 6: Native PAGE analysis of esterase from clone *E. coli* (pSKA). The protein at different steps of purification was analyzed by 10% native PAGE followed by activity staining by fast blue RR salt. Lane 1: *Bacillus* sp. SK320 supernatant, Lane 2: clone *E. coli* (pSKA) supernatant, Lane 3-5: Q-Sepharose A1, A2, A3, Lane 6-12: G-75 A1a, A1b, A2a, A2b, A3a, A3b, A3c

and A3c, respectively (Fig. 6). The enzymes exits as a monomer and this is in contrast to that reported by Takimoto *et al*. (1994), where the *Bacillus subtilis* enzymes are multimeric in nature with the molecular weights ranging from 150 to 280 kDa.

Esterases coded by *srfA* gene of *Bacillus* sp. SK320 purified and characterized in this study, shows biosurfactant activity and also exhibits multiplicity. This is the first report of an esterase with biosurfactant activity in *Bacillus*.

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