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Eco-Friendly Industrial Applications of Metabolites Produced by an Extremely Halophilic Bacterial Strain KV003

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ABSTRACT

Extremely halophilic bacterial strains were isolated from dry fish samples, collected from Malabar Coast (Chavakkad beach). Strain KV003 was identified as Alkalibacillus sps by the employment of 16S rDNA sequencing. It was then submitted in NCBI under the accession number KC920817. Biomass production was optimized which suggested that strain had high affinity towards halo and alkaline environment whereas the optimum growth conditions were 20% NaCl and pH 10 respectively. Study on extracellular enzymes confirmed the production of cellulase. Enzyme kinetic study revealed that secreted cellulase had its parental quality of catalysing reactions in high alkaline condition. Analysis along with commercial detergent reported the compatibility of enzyme to use as an additive in laundry industry. It was significant to notice that enzyme could work in bio stoning of jeans fabric in a controlled manner. Enzyme also had its significant role in bleaching of cotton cloths. Strain KV003 has also been studied for its biosurfacting ability. Production of bio surfactant was confirmed and was used as bio fertilizer whereas the addition of bio surfactant improved growth of plants compared to normal water.

KEYWORDS : *Alkalibacillus* sps, Bio-surfactant, Cellulase and Detergents

INTRODUCTION

Microbial metabolites have always drawn a considerable attention due to its vast biotechnological applications. Metabolites are produced during stationary phase of microbial growth and show various functions such as signalling, catalytic actions etc. They are broadly classified into primary metabolites and secondary metabolites. Primary metabolites will directly involve in growth and regulation of an organism whereas secondary metabolites does not directly involve in those (Demain and Arnold, 1980). It is evident that metabolites reflect the characters of its parental organism, for instance Taq polymerase obtained from extreme thermophilic bacteria, *Thermus aquaticus* which play a vital role in PCR is highly thermostable. Organisms surviving in extreme environments such as high temperature, low temperature, acidic environment, alkaline environment and hypersaline environment etc, which were considered to be hostile to any other organisms, are called as extremophiles. Extremophiles those dwell in hypersaline environments are called as halophilic organism (Vreeland *et al.,* 1980) and could be classified into three classes; Slightly halophilic organisms, Moderately halophilic organisms and extreme halophilic organisms based on its salt requirements (Ventosa *et al.*, 1998). Halophilic organisms have proven to be rich source of metabolites with promising biotechnological applications such as halozymes, biosurfactants, bacteriorhodopsins etc (Margesin and Schinner, 2001). Extracted bioproducts have the advantage of working in high saline environments that make them superior over conventional counter products (Burg, 2003).

Halozymes, the enzymes isolated from halophiles are now replacing chemical catalysts in many industries. Halozymes include proteases, lipases, cellulases, amylases, lipases etc. Cellulase is a group of hydrolytic enzymes that hydrolyze cellulose including exoglucanase (FPase), endoglucanase (CMCase) and β-glucosidase (cellobiase) (Matsui *et al.,* 2000). Cellulases are a very important industrial enzyme as it is used in the textile industry, food industry and paper industries (Kirk *et al.,* 2002). Biostoning is one thrust area in textile industry where cellulase enzyme could be exploited. Conventionally Biostoning is achieved by washing cloths along with small stones. This trivial method has its own disadvantages, as the process is unregulatable and results can be uneven. However the application of cellulase enzyme in bio stoning provides uniform and desired results (Cherry and Fidantsef, 2003). Biosurfactants are surface active compounds which change the surface tension of hydrophobic water-insoluble substrates and thereby enhancing their bioavailability and the rate of bioremediation (Pekdemir *et al*., 1999). Biosurfactants have a dominant hand over their synthetic surfactants due to its structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity. Biosurfactants are hence widely used in food, cosmetics and pharmaceutical industries (Thanomsub *et al*., 2004). In this present study halophilic bacterial strains were isolated and screened for its ability to produce cellulase and biosurfactant production and evaluating its biotechnological and commercial applications.

MATERIALS AND METHODS

Enrichment and isolation of Halophilic bacterial strains

Dry fish samples, collected from Malabar Coast were selected and cut into small pieces of about 2 cm^2 and were inoculated into halophilic broth containing 200 g NaCl; 7.5 g Casaminoacid; 10 g Yeast extract; 3 g Tri sodium citrate; 2 g KCl; and 20 g MgSO .$7H_2O$ in 1000 ml distilled water. The pH was adjusted to 7.0 before autoclaving. Enrichment cultures were subcultured several times under the same conditions. Different colonies were picked and restreaked several times to obtain pure cultures. The pure cultures were stored at -20°C in the isolation medium supplemented with 50% glycerol (Vijayanand *et al*., 2012).

Screening of Cellulolytic Bacterial strains

CMC agar plates were prepared by the addition of 1% Carboxy methyl cellulose to the Halophilic agar. Isolates were streaked onto the surface of the media and then incubated at 40^0C until visible colonies appear. Following incubation, the petriplates were flooded with 0.1% Congo red solution and left as such for 20 minutes. Then the plates were eluted with 1M NaCl. Clear halo zones against the red Congo red background indicated positive result (Upadhyaya *et al.,* 2012).

Phenotypic and Molecular Identification of the isolate KV003

Phenotypic characters of the superior cellulolytic strain KV003 such as pigmentation, colonial elevation, diameter, margin, opacity and consistency were studied (Oren *et al,* 1997). Cell morphology was examined by light microscope of exponentially growing liquid culture. Gram staining was done by taking acetic acid fixed samples (Dussault, 1955). Motility test was also carried out (Vreeland, 1993).Biochemical tests of isolate were carried out according to Bergey's manual of Systematic Bacteriology. 16s rDNA sequencing was employed for the molecular identification of isolate using genomic DNA as template for PCR. The amplicon was sequenced in ABI 3500 XL Genetic Analyzer. Thus obtained sequence was subjected for BLAST analysis as well as

phylogenetic analysis by neighbor joining method using Weighbor (William *et al*., 2000**)**.

Optimization of Culture Conditions for Bacterial Growth

The effect of temperature, pH NaCl Concentrations and agitation levels on the bacterial growth was studied. This was carried out by cultivating the isolate at different temperatures (25°C-55°C), different pH values (pH4.0-11.0), different concentrations of NaCl (1.0-5.0M) and different agitation speeds (0-250 rpm).

Quantification of Cellulase Activity

The reaction mixture contained 50 µl 0.5% CMC (Sigma) in 100 mM sodium acetate buffer (pH 4.0) and 50 µl crude enzyme solution. After incubation at 40ºC for 30 min, 100 µl di nitro salicylic acid reagent (DNS) was added, and the mixture was placed in a boiling-water bath for 5 min and then diluted to 1 ml. Absorbance was measured at 540 nm and one unit of the activity was defined as the quantity of enzyme releasing 1 µ mol reducing sugar per min at 40ºC (glucose as a standard) (Li *et al*., 2006).The protein concentration was estimated by Lowry method using Bovine Serum Albumin as the standard.

Detergent compatibility and Biostoning Activity of KV003 Cellulase.

Stability of KV003 Cellulase in the presence of detergents was investigated by incubating 2 ml of the crude enzyme in 1% (w/v) of detergent solution for 30 min at 40ºC. Control was made by substituting detergent solution with distilled water. The liberation of reducing sugar from the substrate was then evaluated (Iqbal1 *et al*., 2011). Brand new fabric was made to 4cm X 6cm pieces, one fabric piece was incubated with 5ml of the crude enzyme for 30 minutes where as, the other piece was incubated with distilled water (control). Following incubation time, the stoning activity was monitored.

Biosurfactant production and its Partial purification

Cellulolytic strain KV003 was inoculated in the above mentioned halophilic broth medium and incubated at 40^0 C for 14 days at 200 rpm on orbital rotary shaker. After incubation, the broth was centrifuged at 8000 rpm for 20min and the resultant supernatant was subsequently subjected for the screening of Biosurfactant activity such as oil spreading test (Soudi *et al.,* 2009), Drop collapse test (Bodour *et al* .,2004**)** and Emulsification index (E24) was calculated according to Abouseouda *et al.* (2007). Biosurfactant was precipitated by adjusting the pH of the cell free broth to 2.0 using 6 N Hydrochloric acid and keeping it overnight at 4°C. The precipitate thus formed was collected by centrifugation (10,000 rpm, 20 min; 4°C) and dissolved in distilled water (Abouseoud *et al*., 2007).

Influence of Biosurfactant in plant growth.

Ten healthy seeds of *Phaseolus mungo* were selected to investigate the impact of biosurfactant on various plant growth parameters. Five seeds were treated with 2 ml of partially purified biosurfactant purified from *Alkalibacillus salilacus*. The rest of the seeds were treated with distilled water alone (control). After 4 days of incubation, the plants were uprooted and checked for the growth parameters such as height of the stem and length of root.

RESULTS AND DISCUSSION

Isolation and Molecular Characterization of Strain KV003

Dry fish, fish and fish products including pickles, sauces etc have been proved to be an excellent source for the isolation of halophilic bacterial strains. Successful attempts have been reported earlier of using dry fish as a source of halophilic organisms, For instance, Anisha *et al*. (2012) reported the presence of lipolytic halophilic bacterial strains in commercially available dry fish. In the present study, a halophilic bacterial strain KV003 was isolated from dry fish samples. Probably the bacterial contamination in dry fish could be from its hypersaline habitat or from the salt used to process them. By 16S rDNA sequencing and phylogenetic tree view (Figure 1), the strain was found to be most similar to *Alkalibacillus salilacus*; HBCC-3 (Gen Bank entry: EU377478). The next closest homologue was found to be *Alkalibacillus sp.* HS20D (Gen bank entry: EF517966). Morphological and cultural characters of the strain are shown in Table 1. The Sequence was then submitted to NCBI with the accession number KC920817.

Optimization of Cultural Conditions

The optimum cultural conditions for maximizing bacterial biomass were investigated and the results were shown in Figure 2-6. The isolate showed a life span of about fourteen days in which maximum growth was observed on tenth day, after which there was a decrease in viable cell count, suggesting that strain entered its decline phase. 40ºC was found to be the most optimum temperature for maximizing bacterial growth. Shaking conditions enhanced biomass production, with optimum being 200rpm. Similar results were recorded by Vidyasagar *et al*. (2006) and Vijayanand *et al.* (2010). Interestingly the isolate exhibited remarkable tolerance towards salinity (20%) and alkalinity 10 pH. To survive in such extreme conditions nature demands a whole lot of modification in cellular physiology of an organism. Halophiles have accomplished this by one among the three strategies such as osmoregulation, salt in strategy and compatible solute method (Oren, 2000).

Cellulase Assay and Its industrial application

To assess the potential of the isolate KV003 to secrete cellulase enzyme, isolate was grown in CMC containing media and flooded with Congo red. The clear zone obtained confirmed the extracellular secretion of cellulase (Figure 7). Maximum specific activity was observed on ninth day of incubation after which there were a slight decrease in the specific activity (Figure 8). This phenomenon could be due to the excess accumulation of proteins in the culture media which might had negatively regulated the specific activity. Similar reports were made by Verma *et al.* (2012) and Lin *et al*. (2012) on their studies on cellulase enzyme produced by *Bacillus sps*.

Biotechnological applications of KV003 cellulase was investigated by treating jeans cloth with enzyme. The cellulase treated fabric resulted in uniform stoning effect and without undesirable tearing (Figure 8). The enzyme was found to be compatible with detergent without denaturation (figure 10) which will enable it as a suitable ingredient in detergent industries. Alkali tolerant capacity of strain would have made a great contribution for enzyme to sustain its identity. The compatibility of microbial derived cellulase with detergents have been previously reported by Iqball *et al.* (2011) where cellulase produced by*Trichoderma viridae* had been proved its detergent compatibility.

Application of Biosurfactants

Biosurfactant production of the isolate was screened by oil displacement test, Drop collapse test and by calculating emulsification index. A clear space was found in oil displacement test whereas the flat drop (collapsed drop) was seen in drop collapse test, both indicating the positive results of biosurfactant production. The emulsification index was found to be 63%. Preliminary screening results are mentioned in Table 2. Tambekar *et al.* (2012) reported the production of bio surfactant from an extremophile isolated from alkali Lonar Lake. Biosurfactant showed an immense influence in plant growth (Table 4). Biosurfactants would have decreased the surface tension of water and as a result more nutrients would have dissolved in it leading to hiked growth in treated samples.

Figures and Tables



Fig 1.Phylogenetic view of strain KV003



Fig. 7. Congo red staining of plate

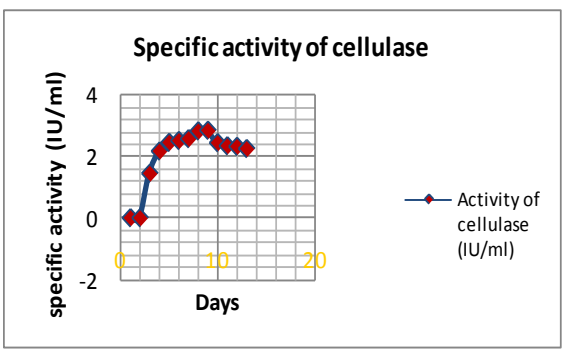


Fig.8. Specific activity of cellulase.

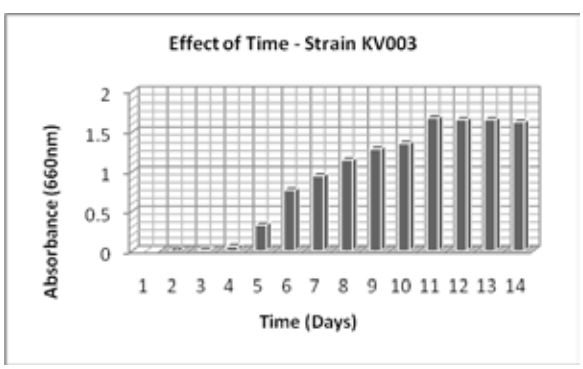


Fig. 2 Effect of time in bacterial growth

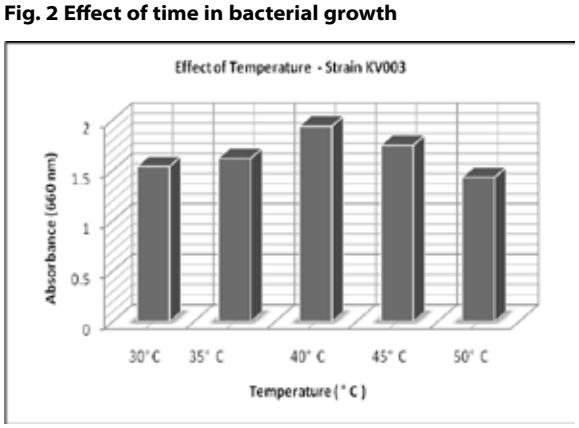


Fig. 3 Effect of temperature in bacterial growth

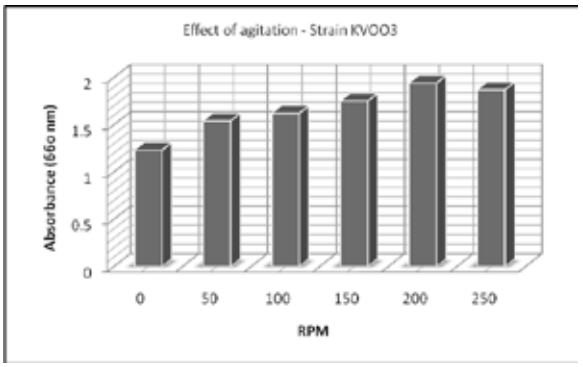


Fig. 4 Effect of agitation in bacterial growth

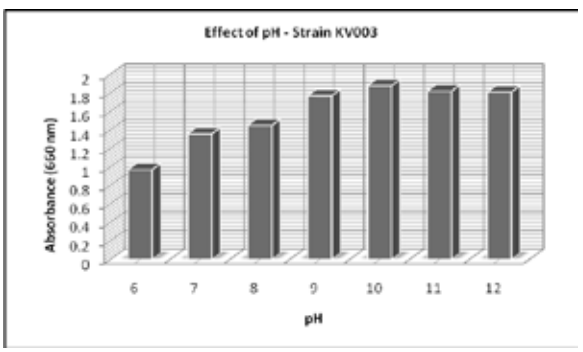


Fig.5 Effect of pH in bacterial growth

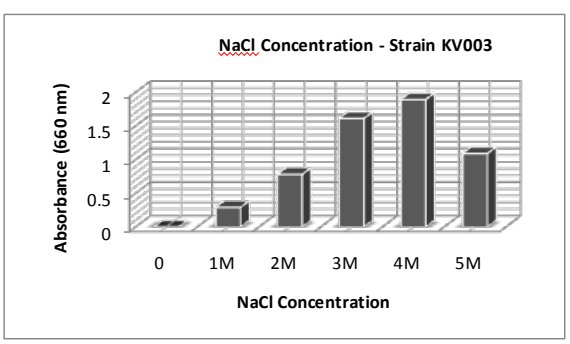


Fig.6 Effect of Salt concentration in bacterial growth



Fig.9 Biostoning activity of enzyme.

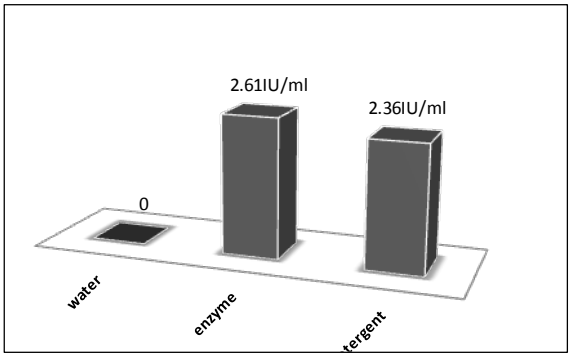


Fig.10 Detergent compatibility of enzyme

Table.1 Characters of Strain KV003

Properties	Strain KV003
Cell Shape	Rod
Cell motility	+
Colony morphology	Circular
Elevation	Convex
Edge	Entire
Density	Opaque
Size	2.0 mm
NaCl tolerance	4- 27 %
pH tolerance	6-12 pH
Oxidase test	+
Catalase test	+
Amylase activity	_
Protease activity	+
Cellulase Activity	+

Table.2 Preliminary biosurfactant screening of isolate

Isolate	Oil displacement test	Drop collapse test	Emulsification index
StrainKV003	+	+	63%

Table 3. Influence of biosurfactant in plant growth

Parameters	Treated Soil	Untreated Soil
Length of Root	27 mm	34 mm
Increase in Length in respective to Control	_	7
% of Increase	_	25.92 %

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