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Full Length Research Paper

Characterization and optimization of agarase from an estuarine *Bacillus subtilis*

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A bacterium producing agarase was isolated from the vellar estuary and potential strain was identified as *Bacillus subtilis*. The ideal parameters was found to be of pH 8, temperature of 40°C and 3% of NaCl concentration for optimal growth and agarase activity. Agar as carbon source and yeast extract as nitrogen source was found to be suitable for optimum growth and agarase activity. The activity of enzyme obtained from the cell free filtrate was 15.2 U/L and the activity of the partially purified enzyme was 5.3 U/L and the purified enzyme activity was found to be 2.3 U/L. The stability of the partially purified enzyme on pH profile was found to be pH 8 and thermostability was found to be up to 40°C. The purified enzyme was determined to be homogeneous on the basis of SDS PAGE and had a molecular weight of 20 KDa.

Key words: Agarase, *Bacillus subtilis,* optimization, stability, SDS PAGE.

INTRODUCTION

Agar, a mixture of polysaccharides extracted from the cell wall of red algae (*Rhodophyta*), especially the genus of Gracilaria and Gelidium. Agar, a cell wall constituent of many red algae (*Rhodophyta*), exists in nature as a mixture of unsubstituted and substituted agarose polymers that form an agarocolloid gel (Craigie, 1990). Agar, a renewable complex polysaccharide composed of agarose and agaropectin, used in preparation of microbial growth media, candy and agar jelly. Agar degradation used in potent supply of biomass, as a source of oligosaccharides, purification of bioactive substances from seaweeds thus the enzyme, agarases which inhibits the growth of bacteria, slows down the degradation of starch, exhibits anticancer and antioxidative activities and a moisturizing and whitening effect on skin and melanoma cells (Kobayashi et al., 1997). Due to these characteristics, agarases have potential applications in food, pharmaceutical and cosmetics industries. Agarases, used to prepare protoplasts of marine algae (Araki et al., 1998) and to recover deoxyribonucleic acid (DNA) from agarose gel (Sugano et al., 1993). Agarolytic bacteria divided into two groups according to their effect on solid agar. Bacteria in group I soften the agar, forming depressions around the colonies, in group II cause extensive liquefaction of the agar (Kobayashi et al., 1997). So far, several agarases isolated from different microorganisms, including Alteromonas (Kirimura et al., 1999; Wang et al., 2006), Pseudoalteromonas (Vera et al., 1998), Vibrio (Araki et al., 1998), Cytophaga (Duckworth et al., 1969) and Acinetobacter (Lakshmikanth et al., 2006). On the other hand few non-marine agar degrading bacteria have also been isolated from soils (Stainer et al., 1942; Sampietro et al., 1971; Hunger et al., 1978), rivers (Van et al., 1974; Agbo et al., 1979) and sewage (Hofsten et al., 1975). Hosada et al. (2003), reported agar degrading *Paenibacillus* sp. from Rhizosphere of spinach. The enzymatic breakdown of agar done by two types of agarases, -agarases and - agarases and they differ based on pattern of hydrolysis of the substrates. The -agarase cleaves the -L-(1,3) linkages of agarose, produce oligosaccharides of the agarobiose series with

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Abbreviations: DNA, Deoxyribonucleic acid; **NA2,** neoagarobiose; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

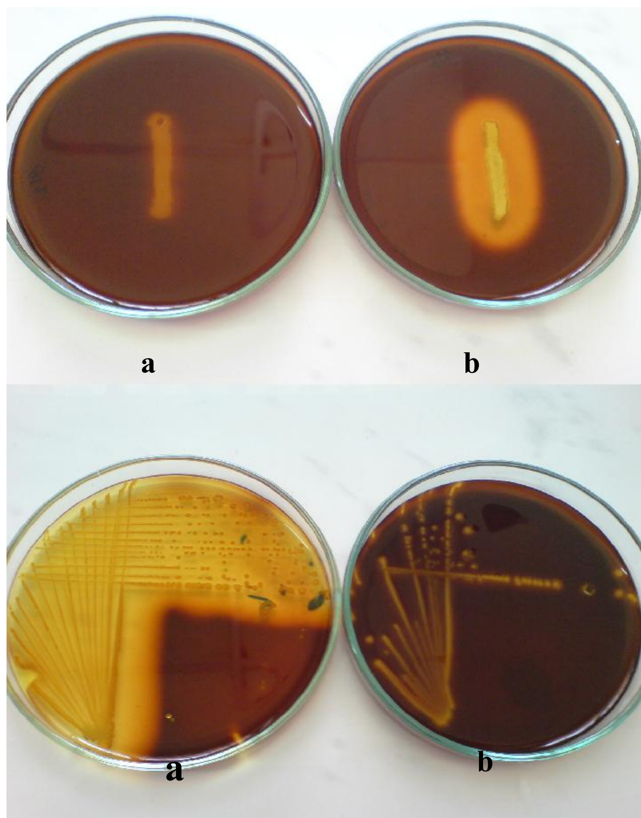

Figure 1. Screening of agarolytic activity a- Control b-agarolytic activity of *Bacillus subtilis.*

Table 1. Biochemical identification of the potential agarolytic strain.

Test	Results
Gram 's staining	+
Morphology	Rod
Catalase	+
Oxidase	+
Indole	_
Motility	_
Methyl red	_
Voge's proskaver	+
Citrate utilization	+
Urease	_
Starch hydrolysis	+
Gelatin hydrolysis	+
Nitrate reduction	+
Spore staining	+

3,6-anhydro-L-galactopyranose at the reducing end, whereas, the -agarases cleaves the -D-(1,4) linkages of agarose, produce neoagaro-oligosaccharides with Dgalactopyranoside residues at the reducing end (Hassari et al., 2001). The ability of degradation of agar or agarose by these agarases makes it widely useful in food, cosmetic and medical industries. The degradation products of agar, the neoagarooligosaccharides inhibit the growth of bacteria and slowdown the rate of degradation of starch. Besides, the neoagarobiose (NA2), a rare compound with both moisturizing and whitening effects on melanoma cells (Kobayashi et al., 1997). The polysaccharide fractions from marine algae by -agarase also have macrophage stimulating activity (Kirimura et al., 1999; Ohta et al., 2004). Most agarolytic bacteria have been isolated from marine habitats but isolations from other sources have also been reported (Hofsten et al., 1975; Van et al., 1974; Hunger et al., 1978).

MATERIALS AND METHODS

Isolation of agarolytic bacteria

For the isolation of agarolytic bacteria, sediment samples from Vellar estuary, Tamil Nadu, India were collected and spread on medium containing 0.05% of yeast extract, 0.25 of peptone and 2% of agar as sole carbon source at a pH of 7.8 and incubated at 30°C for 3 days. Plates were examined for agarolytic activity and assessed by liquefaction or shallow depressions appearing around the colonies. The purity of the isolates was checked by further streaking.

Screening of agarolytic bacteria

For screening of agar degrading bacteria Lugol's solution, which represents potassium iodide, 2 g and iodine, 1 g in 200 ml of water was poured onto agar medium containing 3 day old cultures. Bacterial colonies that formed maximum zone of clearance around the colonies on agar plates were taken for further analysis (Figure 1).

Production of agarolytic enzyme

The potential strain was inoculated into basal medium which constitutes of 0.2% $NaNO_3$, 0.05% polypeptone, 0.05% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.01% CaCl2·$2H_2O$, 0.01% NaCl, 0.002% $FeSO_4{\cdot}7H_2O$, 0.02% $MnSO_4$ (Kim et al., 1993), containing 0.5% agar as sole carbon source, was inoculated with 0.2 ml of the sample and incubated at 30°C for 3 days.

Identification of the potential agarolytic bacteria

The potential agarolytic bacteria were identified based on biochemical characteristics as per the Bergey's manual of systematic bacteriology as showed in Table 1.

Optimization of culture conditions for growth and agarase enzyme production

Effect of pH on bacterial growth and enzyme production

Optimum pH for growth and enzyme production was carried out individually at various pH values ranged from 4 to 9 with an interval of 1 in the basal medium.

Effect of temperature on bacterial growth and enzyme production

The growth of the bacteria culture and its enzyme production was optimized for temperature ranged from 20 to 50°C in the basal medium.

Effect of salinity on growth and enzyme production

The growth of the potential strain and its enzyme activity was optimized in basal medium with different salinity ranged from 10 to 50 ppt with an interval of 10.

Effect of carbon source on bacterial growth and agarase production

Effect of various kinds of sole carbon source such as agar, agarose, galactose and glucose in the basal medium at a concen-tration of 0.5%, were optimized and among these the carbon source which influenced the optimum growth and enzyme production was monitored.

Effect of nitrogen source on bacterial growth and agarase production

Different nitrogen sources such as yeast extract, beef extract and peptone were included individually in the basal medium and monitored the nitrogen source which influenced an optimum bacterial growth and agarase production through activity of the enzyme measured.

Mass scale culture using optimized parameters for enzyme production

Based on the results obtained from the optimization, the mass scale culture of the agarase producing organism was carried out. 1000 ml of production media were inoculated with 1% of inoculum. The fermentation was carried out in 1000 ml Erlenmeyer flasks on a rotary shaker (300 rpm). The biomass and the enzyme activity were tested at every 24 h interval. At the end of the 3rd day the culture was harvested for the recovery of agarase enzyme.

Partial characterization of crude agarase

Partial purification of the enzyme, Agarase by ammonium sulphate precipitation

The culture broth was centrifuged at 15000 x g for 30 min. About 1000 ml of culture filtrate was treated with ammonium sulfate at 70% saturation and the protein precipitated out. The precipitate was collected by centrifugation in a refrigerated centrifuge (15, 000 rpm, 30 mins). The enzyme activity was determined both in the precipi-tated and lyophilized form and assayed under standard conditions. The precipitate was dissolved in 30 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM and dialyzed against the same buffer overnight at 4°C (Hisashi et al., 2003).

Purification of agarase

The clear dialysate was applied onto a Sephadex column (1.5 to10 cm) equilibrated with Tris–HCl (20 mM) buffer pH 7.0. The column was washed with 50 ml of the same buffer to remove the unbound proteins. The enzyme was eluted in 1.5 ml fractions by a discontinuous gradient of NaCl (0–0.5 M) in the same buffer. The fractions with highest agarase activity were pooled and active fraction was used for molecular weight determination.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed with 12% acrylamide gels. The isolated enzyme was run along with the protein marker (Genei pvt ltd, India). Proteins were stained with Coomassie brilliant blue R-250.

Enzyme assay

Agarase activity was measured by the method (Kirimura et al., 1999) under standard assay conditions were portions of 50 µl of enzyme solutions was added 450 µl of 20 mM Tris- HCl buffer (pH 8.0) containing 0.1% (w/v) agarose. After incubation at 40°C for 10 min, the reaction was stopped by immersion in boiling water for 10 min and the amounts of reducing sugars released were measured by the Somogyi- Nelson method using D-galactose as a standard.

Protein assay

Protein concentration in the culture supernatant, ammonium sulphate precipitate and in active fractions of column chromatography was determined by the Lowry's method with bovine serum albumin as standard and the absorbance was read at 280 nm.

Stability of the enzyme

Stability of the crude enzyme at various pH ranges

The influence of pH on enzyme activity was determined as such one hundred l (10 U/ml) enzyme solution was added to different range of pH buffer (6, 7, 8 and 9) 0.9 ml each separately and for every 30 min the enzyme stability under the conditions of this assay was determined by measuring the residual activity irrespective of their stepwise inactivation.

Stability of the crude enzyme at different temperature

The effect of temperature on the stability of the crude enzyme was assayed under conditions of which the enzyme aliquots, 10µl was heated at different temperature ranging from 20 - 50°C with an interval of 10°C for every 30 min and irrespective of their stepwise inactivation the activity were determined calorimetrically by measuring the amount of sugar present in the incubation mixture.

RESULTS AND DISCUSSION

Isolation, enumeration and identification of potential strain

From the sediment samples 4.8×10^6 CFU/g of total agarolytic bacteria was obtained. Morphologically different colony types showing zone of clearance on agar medium were selected and purified. Pure cultures were

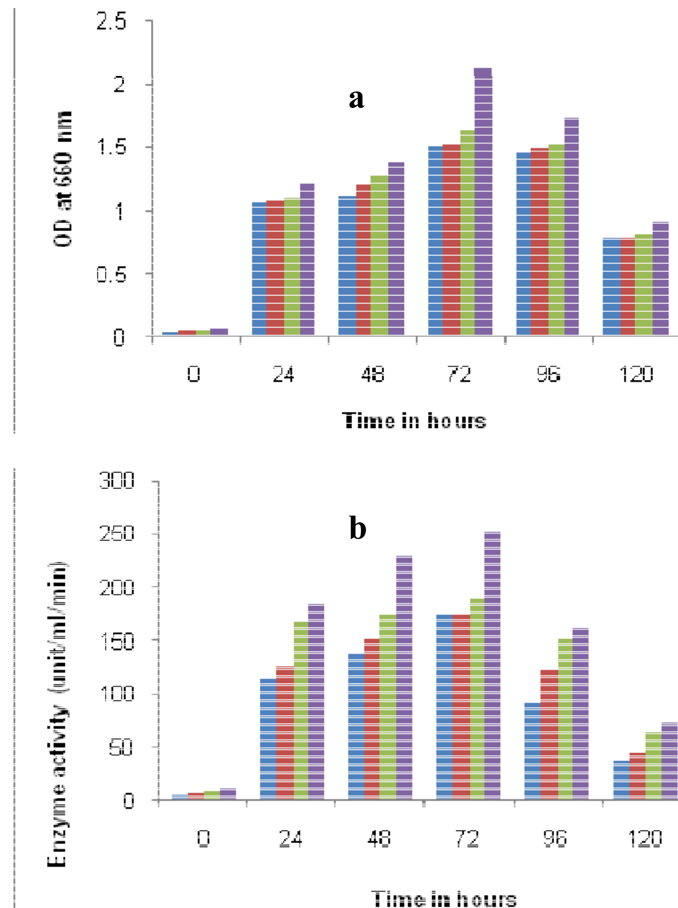

Figure 2. Effect of various pH on growth (a) and enzyme activity (b).

screened for their agarolytic activity by well diffusion method. Among the isolates *Bacillus subtilis* having maximum zone of clearance were subjected to further study.

The above strain was optimized pH 8 was found to be optimum for growth and activity of agarolytic enzyme (Figure 2). The observed result was compared to other agar-degrading bacterial agarase from *Bacillus* sp. which had an optimal pH at 7.6 (Suzuki et al., 2003) According to the report (Lakshmikanth et al., 2006), most of the agarolytic bacteria grew at pH range of 6.5–7.8 and *Pseudomonas aeruginosa* grew at the broad pH range of 5.0–11. It was reported (Hosoya et al., 2009), *P. agarivorans* as a slightly alkali tolerant with pH growth range of 6 to 9 and with an optimum pH of 8 to 9. With comparison of these results indicated that *B. subtilis* as an alkali-tolerant has a specific requirement of sodium chloride for growth and production of extracellular agarases just like most of agarolytic bacteria isolated from marine sources.

From our observation 35°C was found to be an optimum temperature for growth and enzyme production (Figure 3). This effect was reported (Jean et al., 2006) in contrast that two agarolytic Gulf bacteria grew optimally at 25°C and could not tolerate temperature higher than 45°C. For comparison, *T. agarivorans* (Jean et al., 2006) can grow over a temperature range of 20 to 35°C, with an optimum temperature of 25°C and growth of *P. agarivorans* (Hosoya et al., 2009) was observed over a range of 20 to 30°C, with an optimum of 20 to 25°C.

Effect of NaCl concentrations on growth and enzyme activity

From the present observation the optimum NaCl concentration for growth and enzyme production was found to be 3% (Figure 4). A similar was resulted (Sorkhoh et al., 2010) and observed with an optimum of growth and enzyme activity at 3%. As contradictory (Hu et al., 2008) reported a 2.5% of NaCl concentration optimum for the growth and enzyme production.

Effect of various carbon sources on growth and enzyme activity

With the above said growth parameters agar as a sole

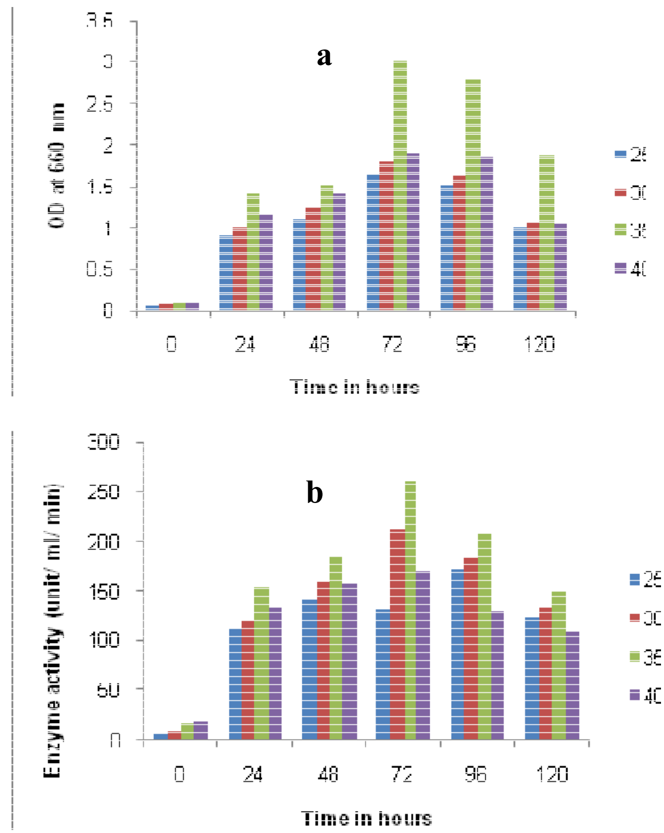

Figure 3. Effect of temperature (25 to 40°C) on growth (a) and enzyme activity (b).

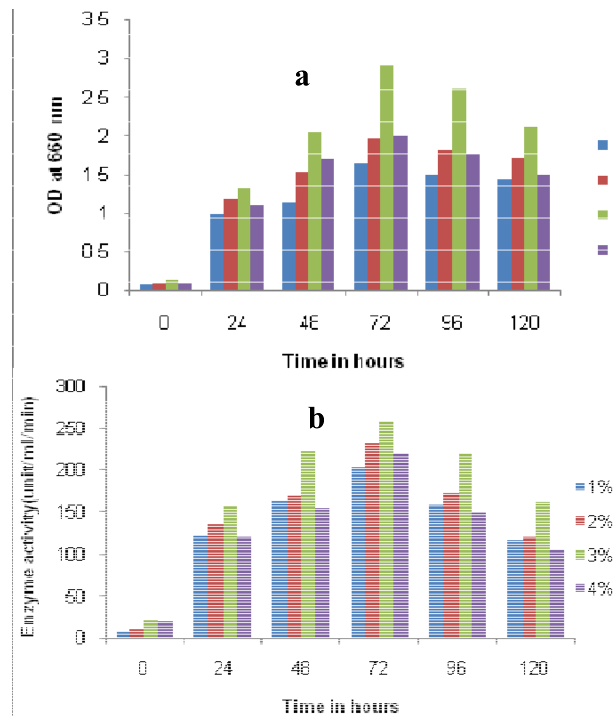

Figure 4. Effect of NaCl concentration on growth (a) and enzyme activity (b).

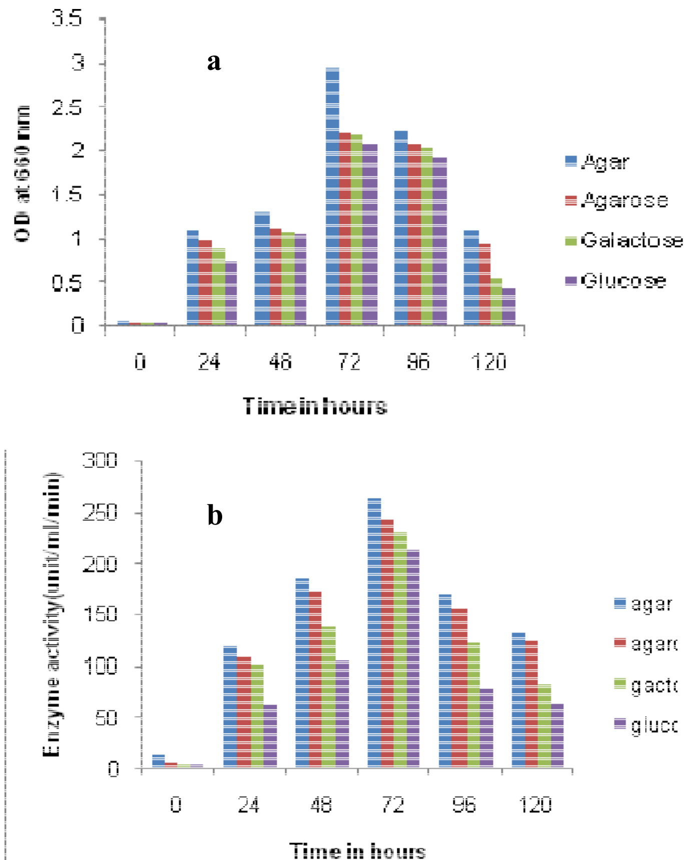

Figure 5. Effect of various carbon source on growth (a) and enzyme activity (b).

carbon source (Figure 5) was preferred as the most preferable substrate for agarase production. Reported in *P. aeruginosa* (Lakshmikanth et al., 2006) and observed an optimum of growth and enzyme production with agar as a sole carbon source and enzyme production decreased when polysaccharides and monosaccharide were used as sole carbon sources.

Effect of various nitrogen sources on growth and enzyme activity

The observed result of the present study indicated that yeast extract as a sole nitrogen (Figure 6) source supported the optimum growth and maximum enzyme production, thus nitrogen was found to be an essential source for the production of the agarolytic enzyme. This effect had been reported with a similar observation using yeast extract as a nitrogen source (Xiao et al., 2009; Lakshmikanth et al., 2006).

Enzyme stability for pH profiles

The pH profile of agarase from *B. subtilis* was found to be a maximum at pH 8.0 (Figure 7). The enzyme was stable under the conditions of this assay was determined by measuring the residual activity at pH 8.0 after 30 min incubation. With evident from (Aoki et al., 1990), that majority of agarases are optimally active in pH ranged between 6.5 and 7.5 and similar results were also observed (Fu et al., 2008). This effect had been studied for *Alcaligenes* sp. (Yu et al., 2009) were the enzyme activity of agarase was relatively stable at pH ranged between 3.0 – 8.5 and decreased sharply as pH raised higher than 8.5.

Stability of the amylase enzyme at different temperature

The thermostability of the purified agarase from *B. subtilis* was found to be up to 40°C (Figure 8) in contrast to the agarases from *P. atlantica* and *Pseudomonas* sp. where the enzyme was rapidly inactivated at temperatures above 30°C was also reported (Fu et al., 2008). The observed effect was contradicted (Lee et al., 2003) in *Alcaligenes* sp. where the agarase enzyme activity was stable between temperature 20 - 35°C and stability decreased sharply as temperature raised higher than

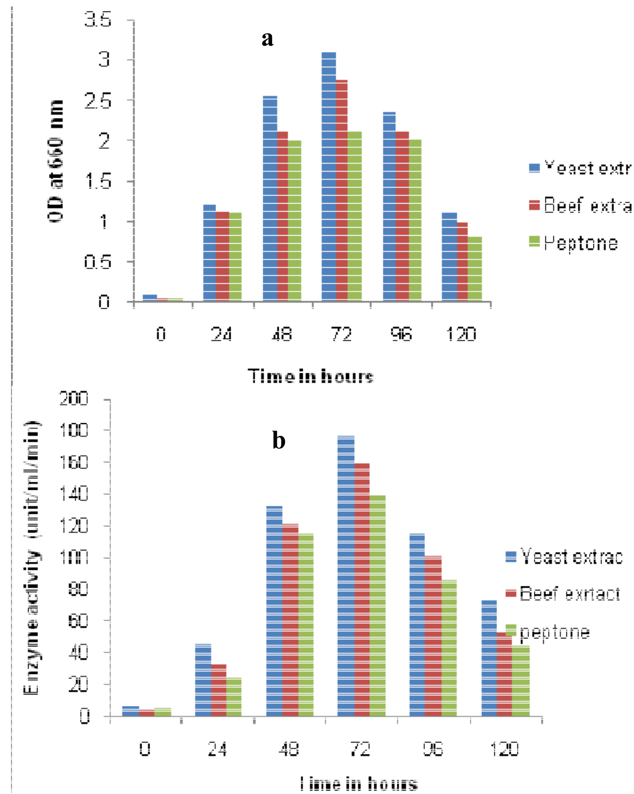

Figure 6. Effect of various nitrogen sources on growth (a) and enzyme activity (b).

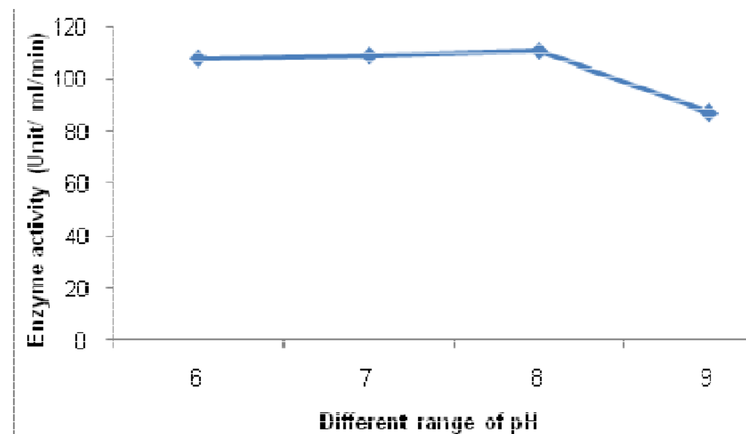

Figure 7. Effect of pH on stability of agarase enzyme.

35°C. The observed result corresponds to the temperature profile of *Alteromonas* sp. with an optimum activity at 40°C and stability of the agarase enzyme up to 40°C (Kirimura et al., 1999).

Mass scale culture

Mass scale culture was done using the ideal parameters and optimized substrates of agar as a sole carbon source and yeast extract as nitrogen source. Agarase activity in the cell free filtrate was found to be 15.2 U/L with protein concentration of 100 mg, activity in the ammonium sulphate precipitate (partially purified) was 5.3 U/L with protein concentration of 32.1 mg and the agarase activity in the active fractions from Sephadex column was found to be 2.3 U/L and protein concentration was observed as 19.0 mg. This result was contradicted by (Suzuki et al.,

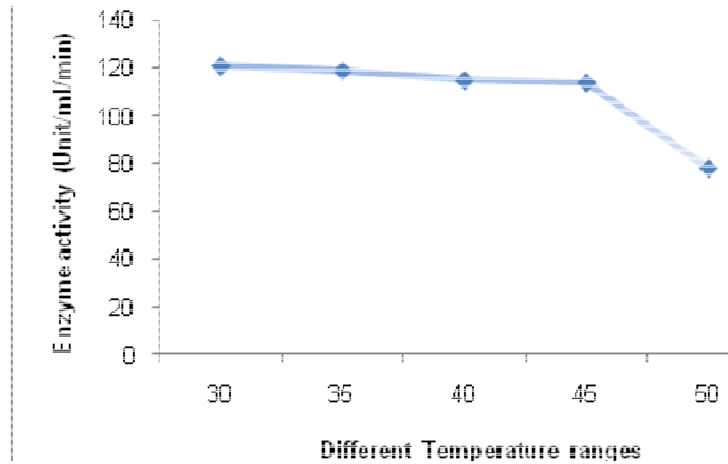

Figure 8. Thermostability of agarase enzyme.

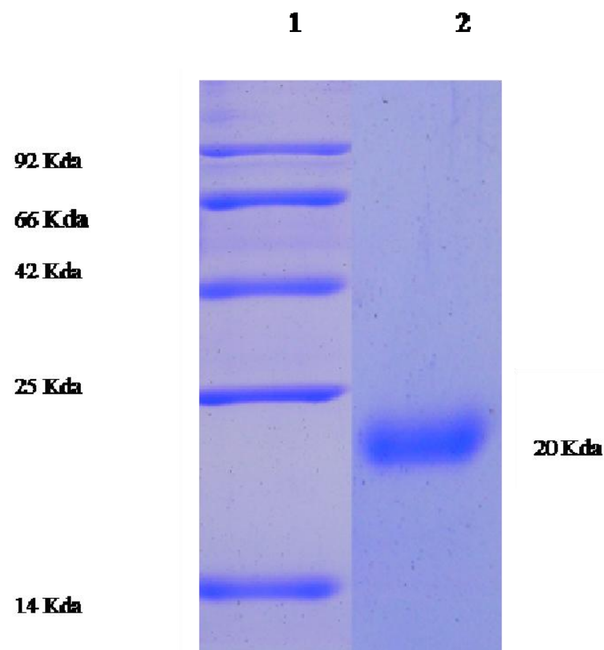

Figure 9. SDS PAGE of agarase enzyme Lane 1 Protein marker Lane 2 purified agarase (20Kda).

2003) 14.1 U of enzyme activity in cell free supernatant with a total protein content of 124 mg, where ammonium sulphate enzyme precipitate had 8.11 U of activity with 42.4 mg of protein content and the purified enzyme from column fraction contained 1.41 U of activity and 0.30 mg of protein content was reported.

SDS PAGE

The purified agarase had a molecular weight of 20 Kda which was close to those reported for agarases, 20 Kda from *Vibrio* sp. (Aoki et al., 1990) and in contrast to the purified agarase reported with molecular mass of 33 kDa, from *Pseudoalteromonas* sp. N-1 (Vera et al., 1998) and *Pseudomonas atlantica,* 32 kDa (Morrice et al., 1983) (Figure 9).

Conclusion

Agarase production by estuarine *B. subtilis* was significantly enhanced by optimization of medium composition and culture conditions. The highest agarase

production of 2.3 U/L was obtained in the medium. The economic medium constitution and culture condition, the high agarase activity, and the dominant occupation of agarase in culture fluid enlighten the potential application of *B. subtilis* for the production of agarase. Thus that agar is a natural product of red algae in the marine environment, and that its presence enhances the bacteria with the combined potential for agar- utilization thus suitable biological tools for self-cleaning and bioremediation of marine ecosystems.

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