

SCREENING, ISOLATION AND IDENTIFICATION OF MARINE BACTERIA FOR AGARASE ACTIVITY AND CHARACTERIZATION OF AGARASE FROM A SELECTED STRAIN

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Agarase is a biotechnologically important enzyme obtained from agarolytic bacteria mostly found in marine environment. Apart from digesting agar-agar, agarose and neoagar-oligosaccharides, few agarases also act on a range of other substrates such as galactomannan, starch, pectin, stachyose and other derivatives of polysaccharides.

Based on mode of cleavage of polysaccharide bonds, two types of agarase have been reported, 1) a-agarase cleaving the a-1,3 linkages of the agar molecules, giving rise to oligosaccharides of agarobiose series with 3,6-anhydro-L-galactose at the reducing end (Young K.S. *et. al.*,1978) e.g., a-agarase from *Streptomyces coelicolor* (Butner *et. al.*,1987), and 2) b-agarase cleaving the b-1,4 linkages, giving rise to the neoagarobiose series with D-galactose at the reducing end (Duckworth and Turvey, 1969) e.g., b-agarase I and b-agarase II from *Pseudomonas atlantica* (Morrice *et. al.*, 1983). The two b-agarases differ in their substrate specificities. b-agarase I degrades neoagarohexaose but not the homologous tetrasaccharides. b-agarase II degrades agar to neoagar-oligosaccharides, of which disaccharide was limiting and predominant
It finds its use in

- extraction of DNA, especially large sized, from agarose gel with higher yields.
- for the preparation of protoplast of plant cells to transform foreign DNA and to make hybrids by protoplast fusion.
- extraction of several useful products such as unsaturated fatty acids, vitamins, carotenoids, betaine etc. from sea-weeds, when used as a mixture of different polysaccharide degrading enzymes.
- for softening the agar media for culturing plant cells.
- for liberating cells and DNA entrapped in agarose films for long term storage.

- for preparing simple oligosaccharides from complex polysaccharides.

Although several agarases have been reported and a few of them are in successful commercial use, and despite India having a large coastal area and huge marine resources, they have hardly been explored for these purposes.

The present study intends to explore our own marine resources for the presence of agarolytic bacteria having useful agarase activity.

The work undertaken in the present study can be summarized and concluded as follows :

- (i) Sea-water samples were collected from the Indian coasts of Vishakhapatnum, Puri and Mumbai.
- (ii) Samples were screened for agarolytic bacteria having superior agarase activity. Four agarolytic bacteria AS2, AS112, 8A and DB₂/D₁ were isolated. Based on qualitative and quantitative screening isolate AS3 was selected for further studies.
- (iii) Agarolytic isolates AS2, AS112, 8A and DB₂/D₁ were characterized, so as to identify their taxonomical positions.
- (iv) Morphological, biochemical and physiological characterization of these isolates revealed that isolate AS3 was different from the other three. Isolates AS112, 8A and DB₂/D₁ had remarkable similarities in biochemical properties. However, they differ from each other morphologically, as observed in electron microscopic studies.
- (v) As a new taxonomic tool, 16S rDNA and gyrB sequence analyses were done and a phylogenetic tree was constructed. The phylogenetic studies based on these molecular approaches showed that isolate AS3 belonged to genus *Colwellia*, and hence the name given to isolate AS3 was *Colwellia sp. AS3*.
- (vi) Isolates AS112, 8A and DB₂/D₁ could not be differentiated by 16S rDNA sequence analysis. gyrB sequence analysis for phylogenetic differentiation also did not reveal much differences among these three isolates. Inter spacer region (ISR) of rrn operon also showed similar pattern when amplified with 16S and 23S rDNA primers.
- (vii) RFLP using 16S + 23S rDNA probe was instrumental in differentiating the agarolytic bacteria in study.
- (viii) The isolates AS112, 8A and DB₂/D₁ were found to belong to the genus *Pseudomonas*, and hence the names given to them were *Pseudomonas agarolytica* AS112, *Pseudomonas agarolytica* 8A and *Pseudomonas agarolytica* DB₂/D₁
- (ix) The extracellular agarases from isolate AS3 were characterized for their enzymatic

properties. The agarases from isolate AS3 were showing highest activity at 45°C temperature and in a pH range of 7–7.5.

- (x) The agarases from isolate AS3 were located on a composite–SDS–PAGE and the sub–unit molecular weights were found to be 32kDa and 47kDa. These two agarases were able to liquefy the agarose.
- (xi) The native molecular weights of agarases from isolate AS3 were determined by gel filtration. It was found that majority of extracellular agarases of isolate AS3 were in dimeric form of 47kDa agarase, whereas 32kDa agarase was in monomeric form. A smaller fraction of 47kDa agarase was also found in monomeric form.
- (xii) These two agarases were electroblotted on PVDF membrane and N–terminus amino acid sequencing was done.
- (xiii) Based on amino acid sequences of 32kDa and 47kDa agarases, degenerate sense primers were designed to amplify the agarase genes through PCR, using genomic DNA of isolate AS3 as the template.
- (xiv) Homology search of N–terminal amino acid sequences of 32kDa and 47kDa agarases with agarase sequences from other organisms helped in designing anti–sense primers to amplify the agarase genes , from isolate AS3, through PCR.
- (xv) Partial genes amplified, using PCR, were cloned in pGEM–T easy vector and sequenced in the automated DNA sequencer ABI prism 377.
- (xvi) The partial genes of 32kDa and 47kDa agarases were similar to each other at the amino acid level but differed very little in the nucleotide sequence towards the C–terminus.
- (xvii) The N–terminal amino acid sequences of these two agarases showed difference, but the partial genes of these two agarases were similar to each other. The difference of these two could be known only when the complete coding sequences are known.

The complete coding sequence for these two agarases will be promising in their cloning, expression and successive commercial applications.