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Isolation and Characterization of an Agarase Producing Bacteria from Marine Sediment

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Abstract : A new agarase was purified from an agarolytic bacterium, *Bacillus megaterium*, a novel agar degrading bacterium isolated form marine sediment. The enzyme was purified from the culture supernatant by ammonium sulfate precipitation method and staining of the purified agarase on an SDS-polyacrylamide gel revealed a single band with an apparent molecular weight of 35 kDa. The optimum pH and temperature for this enzyme were 7 and 40°C, respectively. The effect of medium composition on agarase production was investigated in shake flasks. The most suitable carbon source, nitrogen source, and salt sources were agar, yeast extract, and sodium chloride, respectively.
Keyword: Agarase, Agarolytic bacteria, *Bacillus megaterium.*

Introduction

Agar is an abundant biopolymer found in the cell wall of red algae (Rhodophyta), especially the genus of Gracilaria and Gelidium[1] and is widely used as food additive, gelling agent as well as the feedstock of Bioenergy[2]. Agarose and agaropectin are the main components of agar, while agarose is the gelling component and comprises a linear chain of alternating 3-O-linked-(alpha) -D-galactopyranose and 4-O-linked 3, 6-anhydro-(beta) -L-galactopyranose and agaropectin is a derivative of agarose substituted by sulfoxy, methoxy and pyruvate residues[3]. Agar oligosaccharide can be obtained by chemical as well as enzymatic hydrolysis and it shows many biological activities, includes the inhibitory effect on growth of bacteria[4], the whitening effect on melanoma cells[5], anti-oxidation[6], prebiotic effectiveness[7], antivirus activity[8], anti-inflammation[9] and inhibition of lipid peroxidation[10].

Agarases catalyze the hydrolysis of agar, including α-agarases (EC 3.2.1.158) cleave α-L-(1,3) linkages of agarose to produce oligosaccharides of the agarobiose series with 3,6-anhydro-L-galactopyranose at the reducing end and β-agarases (E.C. 3.2.1.81) Cleave β-D-(1,4) linkages of agarose to produce neoagarooligosaccharides (NAOS) with D-galactopyranoside residues at the reducing end[11]. Agarases is mainly classified based on their amino acid sequences into different glycoside hydrolase (GH) families, GH-16, GH-50, GH-86, GH96, GH117 and GH-118 (http://www.cazy.org/fam/acc GH.html). Among this β-Agarases mainly belongs to GH-16, GH-50 and GH-118 families, which produces neoagarotetraose (NA4) & neoagarohexaose (NA6)[12], neoagarobiose (NA2)[13] and NAOS[14] respectively.

Agarolytic bacteria can be divided into two groups according to their effect on solid agar[15,16]. Bacteria in group 1 soften the agar, forming depressions around colonies, while those in group 2 cause extensive liquefaction of the agar. Most of these bacteria were isolated from marine environments, because agar is a polysaccharide produced by marine seaweeds, it is natural that most of the bacteria which are producing

antifouling metabolites[17], antioxidants[18], enzymes like Laccases are inhabitants of marine habitats[19]. On the other hand, few non-marine agar degrading bacteria have also been isolated from soils, rivers, and sewage[20].

There have been reports of agarases from certain marine mollusks and from several bacteria, including *Actinomyces*, *Agarivorans*, *Alterococcus*, *Alteromonas*, *Bacillus*, *Cellulophaga*, *Cytophaga*, *Microbulbifer*, *Pseudoalteromonas*, *Pseudomonas*, *Saccharophagus*, *Streptomyces*, *Acinetobacter*, *Thalassomonas*, *Vibrio*, *Flammeovirga*, *Halomonas aquamarine* and *Zobellia*[21,22,23]. In this study the marine bacterium, *Bacillus megaterium* isolated from marine sediments, purified by ammonium sulfate precipitation and dialysis and we optimized the medium composition, pH and temperature.

Materials and Methods

Sample collection

Marine soil sediments were collected from marina beach, East coast of India in sterile container containing distilled water and kept in a shaker incubator for 5 days for further analysis.

Screening of Microorganism

Screening of agarase producing bacteria was carried out on Marine Agar containing 5% Peptone, 1% Yeast extract, 19.45 % NaCl, 0.16% $NaHCO_3$ and 15% of Agar. The plates were incubated at 28°C for 3-5 days. Colonies that formed clear zones around themselves after being sprayed with Lugol iodine solution were picked out and purified. The isolated colony was inoculated into Marine broth medium and incubated at 28°C for 24hrs stored in 40% glycerin, at −80°C.

Identification of Microorganism

Isolated bacteria were identified by various morphological and Biochemical test on the basis of the criteria described in Bergey's Manual of Systematic Bacteriology. Then organism was further confirmed by 16S rDNA method. The 16s ribosomal DNA (rDNA) was amplified and the products were sequenced with Applied Biosystems 3130xl Genetic Analyzers. Phylogenetic trees were constructed using a Neighborg-Joining method using the MEGA 5.0 program[24].

Analytical methods

The cell growth was monitored by measuring the absorbance of culture fluid at 600 nm. 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. According to the method of Araki with some modification agarase activity was measured by determining the amount of reducing sugar[1]. 50 µl of enzyme solutions was added 450 µl of 20 mM Tris-HCl buffer (pH 8.0) containing 0.1% (w/v) agarose. The reaction was stopped after incubation at 40°C for 10 min by immersing in boiling water bath for 10min. By using D-galactose as a standard, the amount of reducing sugar released were measured by the method of Somogyi- Nelson[25]. One unit of enzyme activity was defined as the amount of enzyme which release 1 µmol of galactose/minute under the standard condition. The protein concentration was determined by the method of Bradford[26].

Effect of carbon, nitrogen sources and salt concentrations

Effect of carbon, nitrogen sources and salt concentrations on agarose production was estimated. The effect of various kinds of the carbon source, i.e. agar, agarose and galactose, each (0.5%) were investigated. Various kinds of 0.5 % nitrogen source, i.e. yeast extract, peptone and ammonium chloride were applied individually to the fermentation medium. Various kinds of 0.5 % salt source, i.e. sodium chloride, calcium chloride and manganese sulfate were applied individually to the fermentation medium.The above cultures were carried out at 25°C. Agarase activity and biomass were determined at every 24 hrs for continuous 5 days. Triplicate experiments were carried out for each treatment.

Purification of agarase:

Precipitation by ammonium sulfate:

Culture fluid was centrifuged at 15,000 rpm for 15 min and the filtrate (crude extract) was fractionated with ammonium sulfate at 70 saturation in 4°C and the precipitate obtained by centrifugation in a refrigerated centrifuge at 15, 000 rpm for 30 mins was suspended in 30 mM Tris-HCI buffer (pH 7.5) containing 0.1 mM EDTA and the enzyme activity and protein concentration were measured.

Effect of pH and Temperature on enzyme activity

The optimum pH of the enzyme was determined with the buffer solutions of different pHs (6-8) and 0.1 ml of partially purified agarase was added to 0.9 ml of a buffer solution which is containing 0.5% agar as a substrate, the enzyme activity and optical density were measured under the standard assay conditions for each pH after the incubation of 0 to 120 hours at 30°C. The effects of temperature for the agarase activity were determined at different temperature (30-50°C) and incubating the enzyme along with the substrate for 0 to 120 hours at the respective temperatures. The enzyme activity and optical density at 600 nm were plotted against the hours ranges from 0 to 120 to determine the optimal temperature for agarase activity.

Results and Discussion

Among the seven microorganism isolated from the marine soil sediment, one strain shows the significant depression around the colonies were picked and purified for further studies. Morphological characteristics were observed under light microscope indicated that the isolated organism was gram positive and rod shaped. It was positive on catalase, starch hydrolysis and Voges Proskauer, the other biochemical properties of the strain described in Table 1

Table-1: Morphological and Biochemical properties of isolated strain

Properties investigated	Results
Gram's Stain	Positive, Rod
Motility	Positive
Indole	Negative
Methyl red	Negative
Voges Proskauer	Positive
Citrate utilization test	Positive
Oxidase	Negative
Catalase	Positive
Starch hydrolysis	Positive

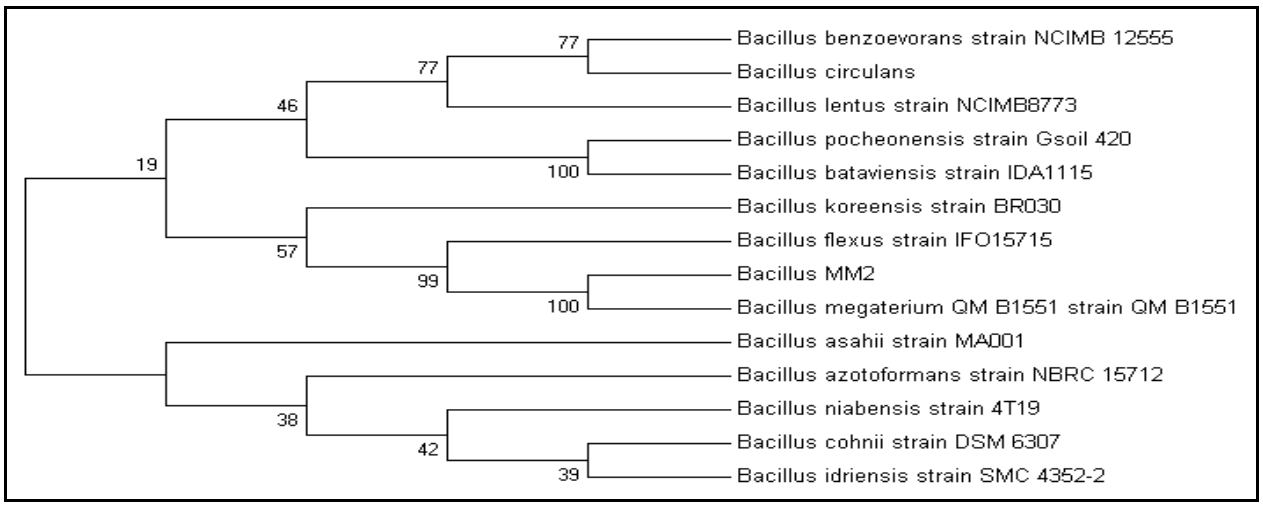


Figure 1- Evolutionary relationship Phylogenic tree

The preliminary identification based on the biochemical and physiological characteristics showed that the strain belongs to the genus Bacillus (Bergey's Manual). Further, the 16S rDNA sequence revealed its 100% homology with bacterium *Bacillus megaterium* from a BLAST search of the NCBI database. The Figure 1. Shows the phylogenetic tree of *Bacillus megaterium* and other close homology microbes. The evolutionary history was inferred using the Neighbor-Joining method [27]. The bootstrap consensus tree inferred from 1000 replicates[28] is taken to represent the evolutionary history of the taxa analyzed[28]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [28]. The evolutionary distances were computed using the p-distance method [29] and are in the units of the number of base differences per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 795 positions in the final dataset. So far only four different agars producing bacillus strains have been reported, they are *Bacillus agarexedens*[30], *Bacillus cereus* [31], *Bacillus* MK03 [32] and *Bacillus megaterium*, in addition to several other bacterial species.

Purification of Agarase enzyme

Ammonium sulphate is mainly used to purify the enzyme because of its ability to neutralize the protein surface charges and to disrupt the water layer surrounding the protein, it decreases protein solubility and increase the protein precipitation[33,34] . In order to concentrate the crude extract of agarase, the supernatant of growth culture of *Bacillus megaterium* was brought to 30-50, 50-70, 70- 90% (w/v) saturation by adding ammonium sulphate. Since the enzymatic activity increased as the saturation increased, the highest activity of the precipitate showed at a saturation of 70-90 % and the precipitate was resolved and dialyzed. The result depicted in Table.2.The precipitate achieved 36.57 mg/ml protein with the enzyme activity 20 units/ml/min and the dialyzed sample achieved 30.65 mg/ml with the enzyme activity 28 units/ml/min.

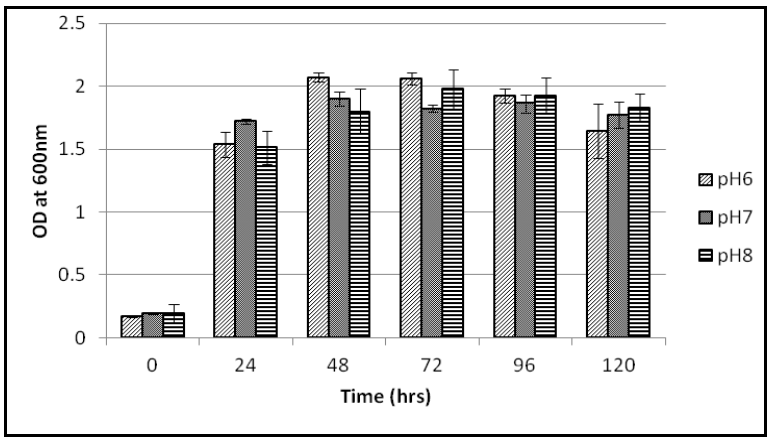
Table: 2 Protein estimation

Sample	Values (mg/ml)
Crude	10.97
30 – 50 %	15.52
50 – 70 %	27.74
70 – 90 %	36.57
Dialysed sample	30.65

Effects of pH and Temperature

The agarase activity and optimal density were measured for different pH and temperatures at different hours revealed that pH 7.0 and temperature 40°C at 72 hours were optimum for the enzyme activity and the results depicted in Figure 2a, 2b, 3a and 3b. Also agarase had high stability in the pH range between 6-8 and temperature ranges between 30 to 50 at 72 hours. The enzyme activity consistently increased till 72 hours incubation, after that drastically decreased for both pH ranges (6-8) and temperature ranges (30-50°C). The optimum pH 7 for agarase activity from marine bacteria *Altermonas* sp., SY 37-12 has been reported[35].Suzuki reported that the optimum pH 6.6 for agarase activity from *B. megaterium* and 7 for *B. cereus*[32]. The pH 7 showed the second maximal activity from the marine sediment bacteria has been reported by Saravanan[36]. The optimum temperature for agarase activity from *Bacillus cereus* ASK202 was 50ºC[31] while the agarases from Agarivorans (40◦C)[37] and it was similar to Pseudoalteromonas antarctica N-1[38].

a)



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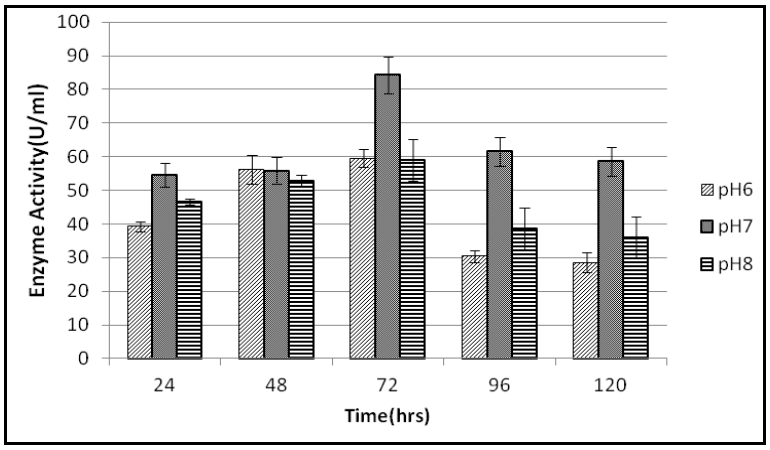
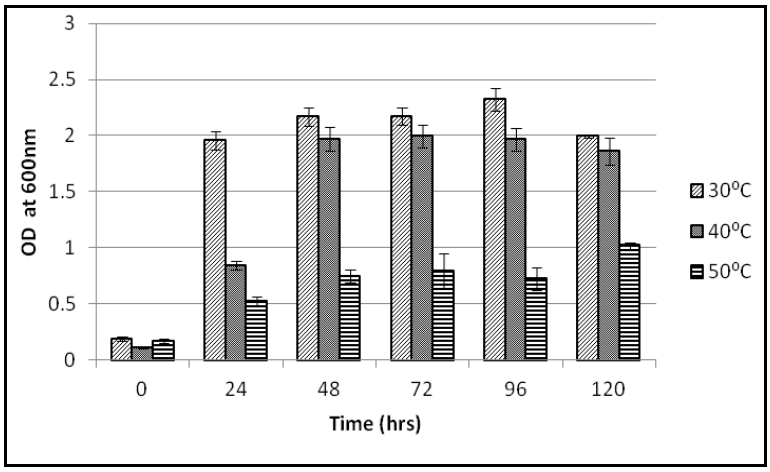


Figure 2: Effect of pH.

a)



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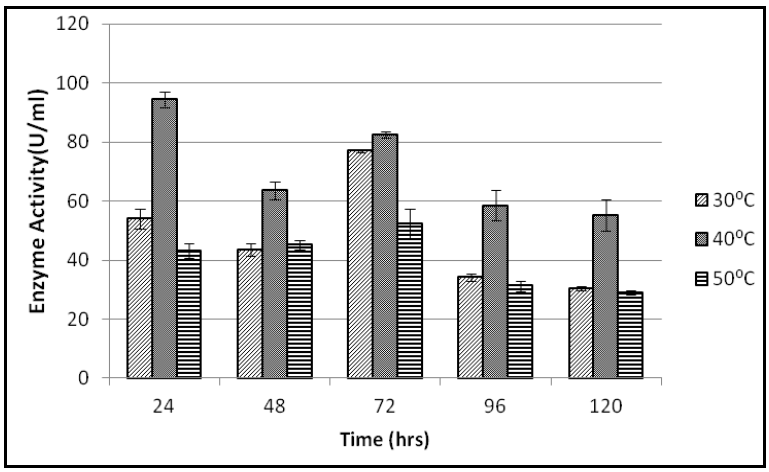
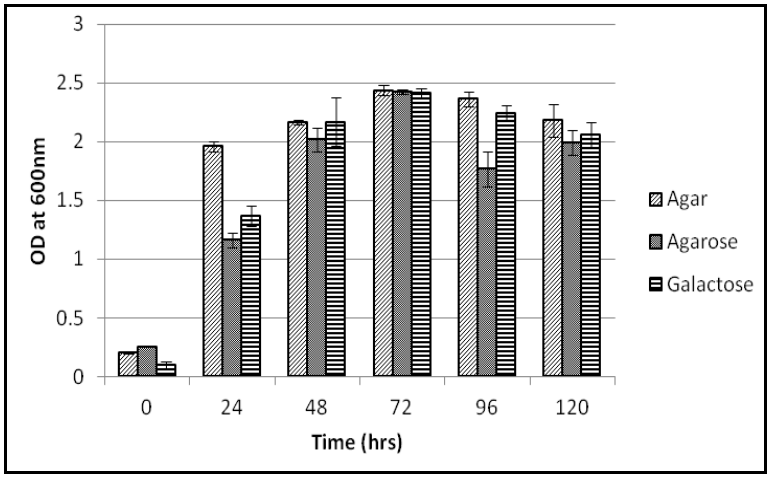


Figure 3 : Effect of Temperature

a)



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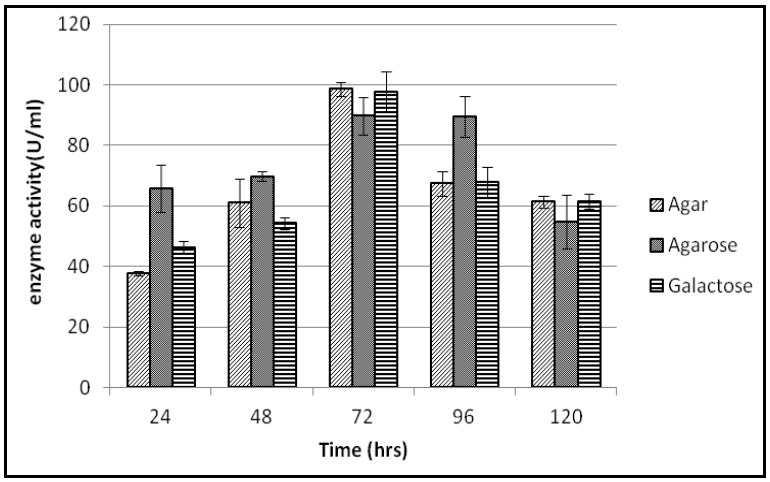
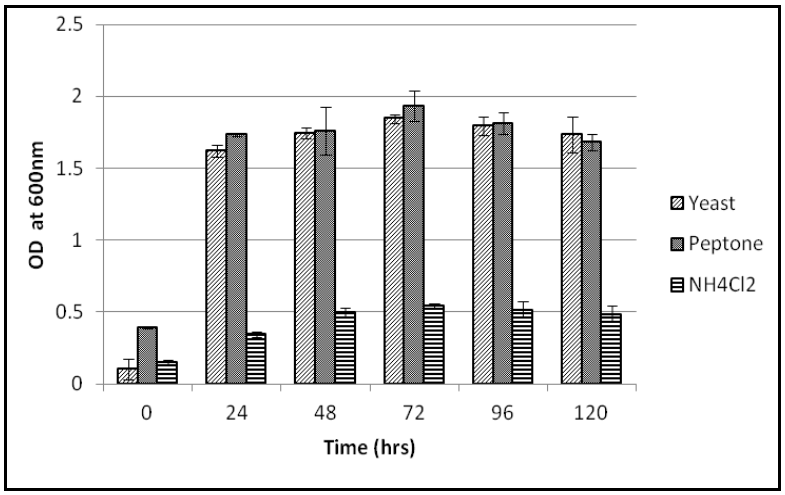


Figure 4: Effect of Carbon source

Effect of Carbon, Nitrogen and Salt sources on agarase production

The effect of various kinds of sole carbon source on agarase production was shown in Figure 4a and b. The maximum agarase production and biomass were obtained when agar was used as the sole carbon source, while the agarase production and biomass were decreased when agarose and galactose used as a sole carbon source. The result indicated that agar was the most effective carbon source for agarase production by *Bacillus megaterium*. The results are similar to reports of Lakshmikanth[39], in which *Pseudomonas aeruginosa* AG LSL-11 produced agarase when agar was used as the carbon source.

a)



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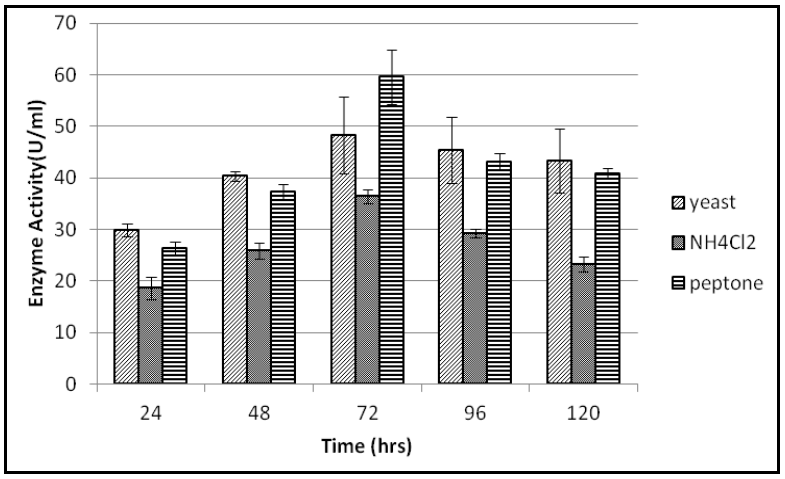
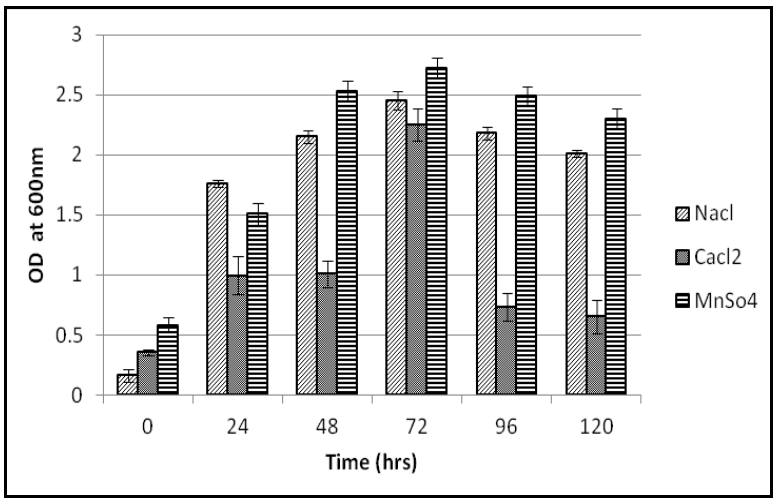


Figure 5 : Effect of Nitrogen source

The effect of commonly used nitrogen source on agarase production by *Bacillus megaterium* is shown in Figure 5a and b. The maximum agarase activity was obtained when the yeast extract was used as the nitrogen source. Agarase activity decreased when organic nitrogen source (peptone) and inorganic nitrogen source (NH_4Cl_2) were used. These results indicated that yeast extract was better than peptone and NH_4Cl_2 for the production of agarase by *Bacillus megaterium*. At the same time, biomass concentration was decreased when peptone and NH_4Cl_2 compared to yeast extract used as the nitrogen source, the result depicted in Figure 6a. The effect of salt sources $NaCl_2$, $CaCl_2$ and $MnSo_4$ for the Agarase activity and biomass concentration were depicted in Figure 6b. Agarase activity and biomass concentration were increased when $NaCl_2$ used as a salt source than $CaCl_2$ and $MnSo_4$..

a)



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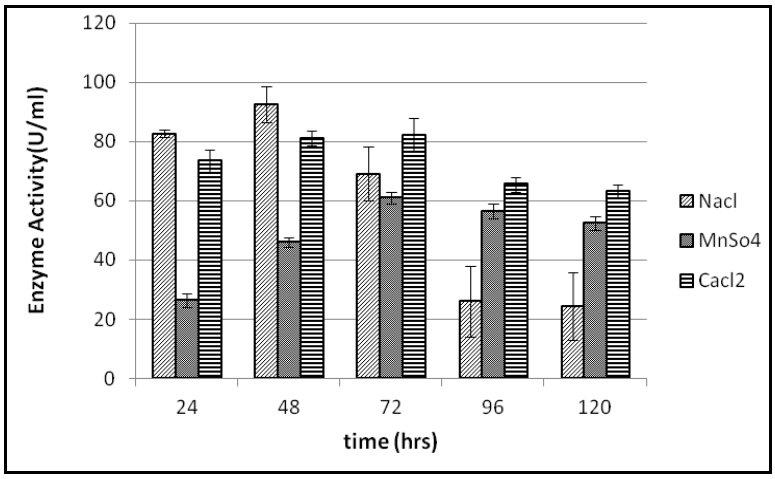

Figure 6: Effect of Salt sources.

Similarly the agarase activity was increased for *P. aeruginosa* AG LSL-11when yeast extract was used as the nitrogen source[16] , for the production of agarase by Acinetobacter sp. AG LSL-1 and Cytophaga flevensis, the optimal nitrogen sources were $NaNO_3$ and NH_4NO_3, respectively[16,11].

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