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RESEARCH ARTICLE

Isolation, characterization and optimization of agarase enzyme from Micrococcus luteus F2

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ABSTRACT

An agar degrading bacterium Micrococcus luteus F2 was isolated from river water. The identification of strain done by partial 16S-rRNA sequencing. This organism produced agarase enzyme and degrade agar at 37˚C, pH 5.0. Highest activity obtained 0.203 U/ml at 37˚C, 0.16 U/ml at 0.5% agar concentration, 0.136 U/ml at pH 5.0, and 0.10 U/ml at 0.09% NaCl concentration. The enzyme agarase produced by this strain was partially purified by ammonium sulphate precipitation and dialysis. It gave 1.14 fold and 1.049 fold purification in 70% and 80% saturation respectively.

KEYWORDS:

Micrococcus, Agarase, Precipitation, Fold purification, Saturation.

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INTRODUCTION

Agar is widely employed as a gelling agent in the preparation of microbiological culture media, and agarose is much used as an anticonvection agent in electrophoresis and diffusion experiments [4]. Some red algae like Gelidium and Gracilaria are known for the location of two major pollysaccharides of agar, namely Agarose and Agaropectin in their cell walls [9]. Agarose (M.W. 100 kDa) is linear chains of repeatedly alternating units of β-1,4 linked-D-galactose and α-1,3 linked 3,6 anhydro L-galactose [2] while Agaropectin (M.W. 20 kDa) is a sulfated polysaccharide in which some hydroxyl groups of 3,6-anhydro-L-galactose residues replaced by sulfoxy or methoxy and pyruvate residues [3]. Agar can be degraded by agarase enzymes that are classified into two distinct types: α-agarases (EC 3.2.1.158) and β-agarases (EC 3.2.1.81) on the basis of their cleavage pattern [1]. The α-agarase break α-1,3 linkages of agarose to produce agaro-oligosaccharides while β-agarase enzyme breaks β-1,4 linkages of agarose to produce neoagaro-oligosaccharides [9]. Agarase enzyme isolated from different microorganisms, including Alteromonas, Pseudoalteromonas, Vibrio, Cytophaga, Catenovulum sp., Microbulbifer, Salegentibacter, Zobellia, Agarivorans, Paenibacillussp., Klebsiella [5, 8] . Agarase enzyme is used to retrieve DNA from agarose gel [11] also used as food additives and gelling agent [3], as anti-oxidants [12], in cosmetics, as moisturizers [6], in medicine industry, as immunity booster [13].

MATERIALS AND METHODS

Isolation of agarase producing bacteria

Sample collection was carried out Tapi river, Causeway, Surat. 50 ml of sterile Medium B (2g $NaNO_3$, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.02g $CaCl_2$, 0.02g $MnSO_4.H_2O$, 0.02g$FeSO_4.7H_2O$, 0.2% Agar in 1000 ml distilled water; pH 7.5) was inoculated with 10 ml water sample and incubated at 37˚C for 48 hours [4]. After incubation it was serially diluted and 0.1 ml aliquot was spreaded from 10^{-2},10^{-4},10^{-6} dilutions on sterile MSA (Mineral Salt Agar) plates and incubated at 37˚C for 24 hours.

Screening of agarase producing bacteria

The plates having isolated colonies were flooded with Lugol's iodine, colonies having clear zone were picked and purified by further streaking [10,7]. Each isolated colonies were studied for its morphological characteristics.

Molecular identification of selected isolate

Molecular identification of selected isolates was carried out by 16S rRNA sequencing. DNA sequencing reaction of PCR amplicon was carried out with primer 27F using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs.

Fermentation

1 ml suspension of each isolates were prepared by comparing it with Macfarland standard 1.0 and transferred into tubes having 5 ml inoculum medium (MS broth) (0.1g $CaCl_2$, 0.5g $MgSO_4.7H_2O$, 0.5g $(NH_4)_2SO_4$, 0.5g K_2HPO_4, 0.5g NaCl, 0.3% Agar, 1000 ml distilled water; pH 5.0) and incubated at 37˚C for 24 hours. After incubation 2% of inoculum was inoculated into sterile MS broth and incubated at 100 rpm on rotary shaker at 37˚C for 24 hours [9]. Clear supernatant was collected by centrifugation at 10,000 rpm for 10 min at 4˚C. Agarase activity was carried out from supernatant.

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Agarase activity assay

The agarase activity was assayed by DNSA (3,5-Dinitrosalicylic acid) method using D-galactose as standard. Standard galactose stock solution (0.5 mg/ml) prepared in deionised water. 1 ml of enzyme (culture supernatant) was mixed with 2 ml of 0.25% agar solution in deionised water, incubated at 37˚C for 15 min. After incubation, 2 ml of DNSA reagent was added and boiled for 5 min at boiling waterbath. 1 ml of 40% Rochelle salt solution was added and cooled down to room temperature and optical densiy was taken by using UV-vis spectrophotometer at 540 nm against blank. 1 Unit of enzyme activity is the amount of enzyme that released 1 µmol reducing sugar (D-galactose) per minute from agar substrate under standard assay conditions [7,9].

Optimization of culture condition

2% inoculum was inoculated into sterile MS broth. Each flask labelled with different temperature such as 20˚C, 30˚C, 37˚C, 45˚C, 55˚C. Five different pH taken were 5.0, 6.0, 7.0, 8.0, 9.0. pH of each tube was set by using sterile 1M NaOH solution or 10% tartaric acid. Various agar concentration such as 0.1%, 0.2%, 0.3%, 0.4%, 0.5%. Varying concentration of NaCl such as 0.01%, 0.03%, 0.05%, 0.07%, 0.09% were taken. Each flask incubated at 100 rpm on rotary shaker for 48 hours. After incubation, clear supernatant of each tube was collected by centrifugation at 10,000 rpm for 10 min at 4˚C. Enzyme activity and protein were measured by DNSA method &Folin-Lowry's method respectively.

Partial purification of agarase

Ammonium sulphate precipitation

100 ml of sterile MS broth was inoculated with 2 ml exponential growth phase culture and incubated on rotary shaker at 100 rpm at 37˚C for 48 hours. After it, the broth was centrifuged at 10,000 rpm for 10 min at 4˚C to remove cell and residual agar gel. The clearsupernatant was collected, solid ammonium sulphate at 70% and 80% saturation were calmly added to the separate tubes having supernatant by constant stirring for an hour, incubated overnight at 4˚C. After this, the blackish-brown sticky precipitates were collected by centrifugation at 10,000 rpm for 10 min at 4˚C. Precipitates were dissolved into 1 ml of 20 mM Tris-HCl buffer (pH 7.0). Enzyme activity was measured before and after precipitation by DNSA method and fold purification was calculated [9].

Dialysis

The precipitated enzyme in 20 mM Tris-HCl (pH 7.0) buffer was dialysed against same buffer with 1:500 dilution into deionised water, and kept overnight at 4˚C. After dialysis, the enzyme activity was checked and protein content also measured.

Measurement of Protein content

Total protein content of culture supernatant was measured by Folin-Lowry's method. To calculate total protein, Bovine serum albumin standard was used [10]. The concentration of BSA stock solution was 200 µg/ml, it was prepared in deionised water.

RESULTS

Isolation and screening of agarase producers

Isolates obtained from MSA medium were screened for agarase production. Screening was done by flooding the plate with lugol's iodine that form clear zone around the colonies while leave the other area into bluish-violet in color. Figure 1 shown the isolation and screening. The isolate showing zone of clearence was named as F2 was selected and it's morphological & colonical characteristics were notedown (Table 1).

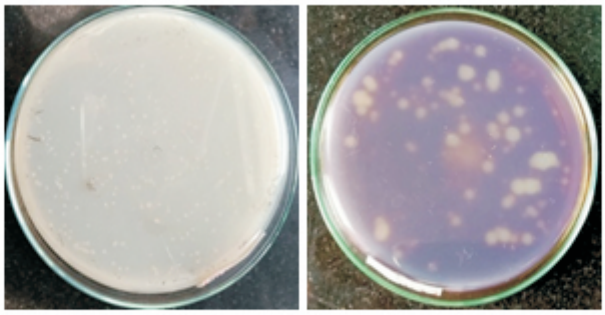

Figure 1. Isolation & Screening of agarase producers

Table 1. Morphological & Colonical characteristics of isolate F2.

Character	Result
Size	Intermediate
Shape	Circular
Edge	Entire
Elevation	Raised
Texture	Smooth
Consistency	Wet
Opacity	Translucent
Pigmentation	Yellow
Gram reaction	Gram positive
Morphology	Cocci

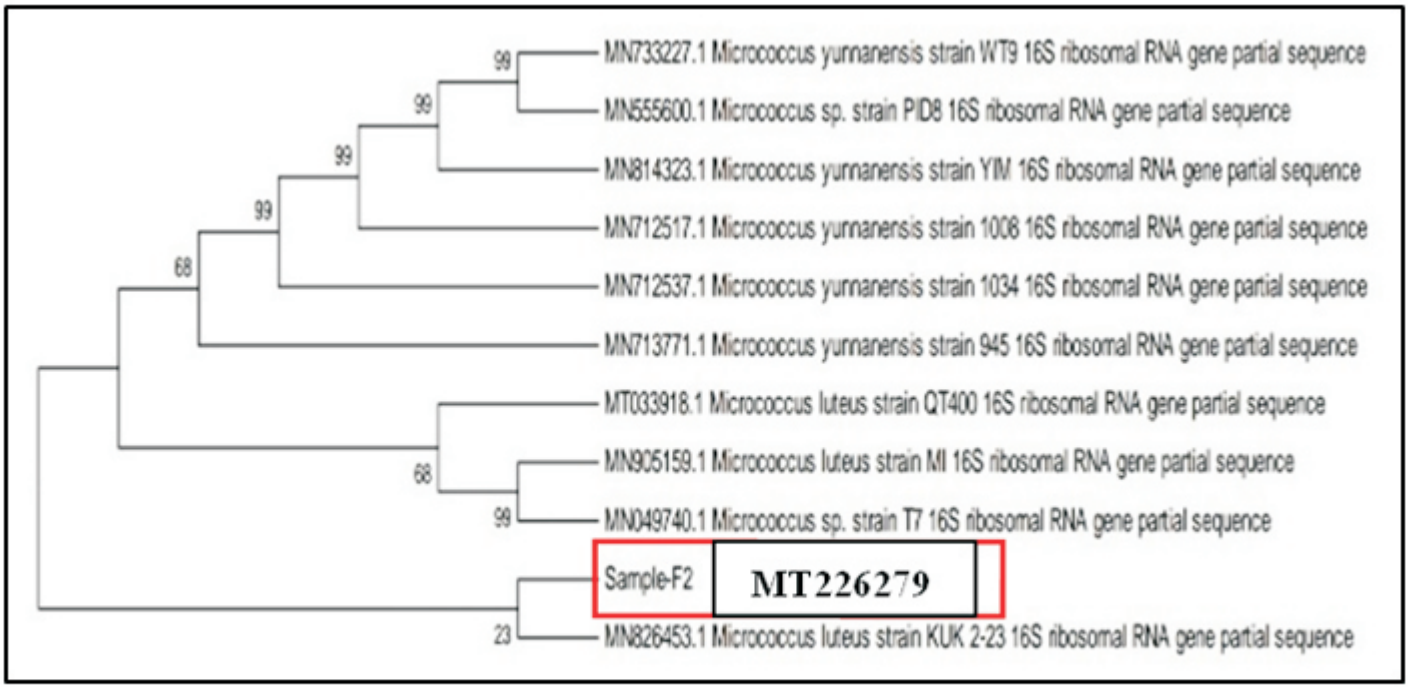

Figure :2 Evolutionary relationship of taxa

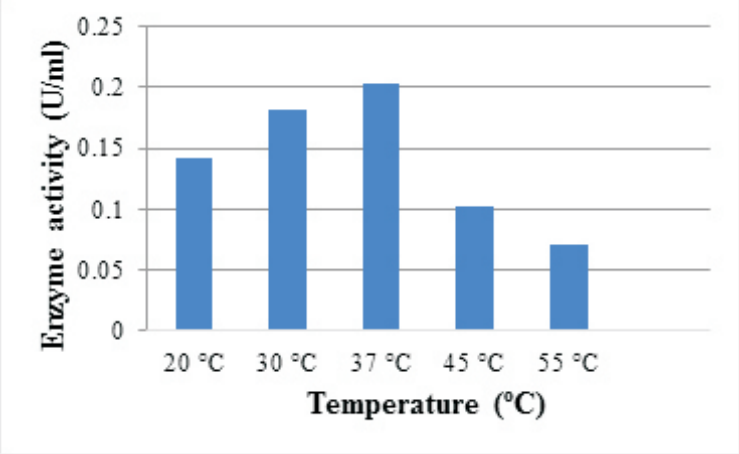

Figure : 3 Effect of incubation temperature on agarase production.

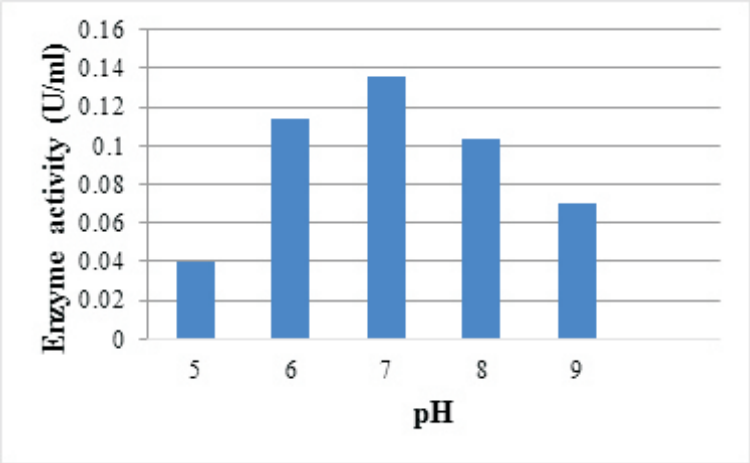

Figure : 4 Effect of pH on agarase production.

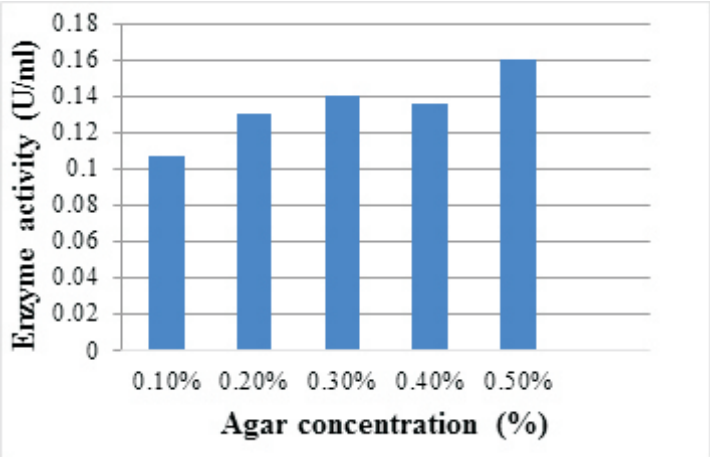

Figure : 5 Effect of agar concentration on agarase production.

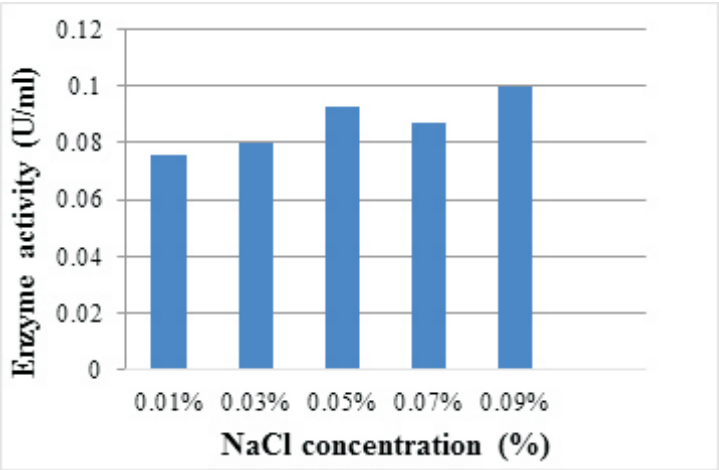

Figure : 6 Effect of NaCl concentration on agarase production.

Molecular identification of isolate F2

By performing the 16S-rRNA sequencing by Sanger dideoxy sequencing method, the selected isolate no.F2 obtained from river water was identified as Micrococcus luteus using BLAST tool of NCBI. The genomic DNA of Micrococcus luteus contain 678 bp.The partial sequence of Micrococcus luteus F2 has been submitted to NCBI with accession no. MT226279. The phylogenetic tree reveals the evolutionary relationship of taxa (Figure 2).

Agarase activity assay

The isolate F2 identified as Micrococcus luteus shown agarase activity 0.44 U/ml after 24 hour of submerged fermentation while it shown specific activity about 5.5 U/mg.

Optimization of culture condition

Different parameters were optimized for agarase production by submerged fermentation.

Effect of incubation temperature

In this study, agarase production was carried out at various temperature and indicated that incubation temperature affect the production of agarase, the enzyme activity at different temperature was recorded and shown that the given isolate F2 shown maximum enzyme activity 0.203 U/ml at 37ºC temperature. Above 37 ºC the enzyme activity decreased that indicated that agarase enzyme may denature above 45 ºC (figure 3).

Effect of pH

pH of the medium highly affects the product stability the selected isolate was grown in medium with different pH ranging from 5.0-9.0, the highest enzyme activity was found 0.136 U/ml at pH-7.0 indicated that the optimum pH for agarase production by Micrococcus luteus was 7.0 pH (figure 4).

Effect of agar concentration

The effect of agar concentration from 0.1%-0.5% was studied on agarase production. The maximum activity was found 0.16 U/ml at agar concentration 0.5% (figure 5).

Effect of NaCl concentration

The effect of salinity on growth and product stability was observed. NaCl concentration ranging from 0.01%-0.09%, the maximum agarase activity found was 0.10 U/ml at 0.09% NaCl concentration (figure 6).

Partial purification of agarase enzyme

Purification of agarase enzyme was carried out by ammonium sulphate precipitation and dialysis methods. 70% and 80% saturation of crude enzyme were carried out then both the concentrated enzymes were dialysed. The specific activity of enzyme with 70% saturation was 2.07 U/mg and fold purification and % yield were 1.14 & 13.7% respectively.The specific activity of enzyme with 80% saturation was 1.90 U/mg and fold purification and % yield were 1.04 & 16.22% respectively. After dialysis the fold purification was increased in case of 80% saturation while it decreased in 70% saturated enzyme. After dialysis the fold purification of 70% & 80% saturated enzymes were 1.094 and 1.35 respectively. Total protein content was measured by Folin-Lowry's method.

DISCUSSIONS

An agar degrading microorganism isolated from river water which was identified as Micrococcus luteus. It was gram positive cocci, aerobic, nonmotile and yellow pigmented organisms which degraded agar by extracellular agarase enzyme. The production of agarase was fluctuated by changes in temperature, pH, agar concentration and NaCl concentration. Mostly agarase enzyme shown its activity between 5-9 pH. This agarase from Micrococcus luteus also shown optimum pH at 7.0. the optimum agar concentration required for hihgest activity was 0.5%. As agar concentration increased the agarase production and cell growth also increased but the gel strength of medium was proportional to the agar concentration. Parasher and kumar (2019) used 1.5% and 2.0% agar concentration for optimization but at this much concentration the gel structure formed and become more difficult for organisms to grow. The optimum temperature was 37ºC as the temperature icreased to 45ºC and 55ºC the agarase activity decreased due to structural deformality.

CONCLUSION

The isolate producing maximum agarase production was Micrococcus luteus which identified by 16S-rRNA sequencing. The optimum condition for agarase production by this strain was examined. The optimum temperature and pH determined were 37 ºC and 7.0 pH respectively. The optimum substrate concentration was 0.5%. The optimum NaCl concentration was 0.9%. This information provides the ideal medium composition for higher production of agarase by this bacterium. The partial purification of agarase by ammonium sulphate precipitation shows the %yield was 13.7% in 70% saturation and 16.22% in 80% saturation. The fold purification was 1.14 in 70% saturation and 1.049 in 80% saturation. After dialysis the fold purification increased to 1.35 fold in 80% saturation.

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