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Agarase Production by Marine *Pseudoalteromonas* sp. MHS: Optimization, and Purification

Mona M. Sharabash¹ Samia S. Abouelkheir^{2*}  Mona E. M. Mabrouk³ Hanan A. Ghozlan¹
Soraya A. Sabry¹

1. Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, 21321, Egypt

2. National Institute of Oceanography and Fisheries (NIOF), Marine Environment Division, Marine Microbiology Laboratory, Egypt

3. Botany and Microbiology Department, Faculty of Science, Damanshour University, Damanshour, Egypt

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ABSTRACT

Agar is an essential polysaccharide that has been utilized in numerous fields. Many kinds of literature have been published regarding agarolytic microorganisms' isolation and agarases biochemical studies. In this search, a local marine agarolytic bacterium associated with marine alga *Ulva lactuca* surface was isolated and identified as *Pseudoalteromonas* sp. MHS. The agarase production was parallel to the growth of *Pseudoalteromonas* sp. MHS as cells displayed a lag phase (2 h), subsequently an exponential growth that prolonged till 10 h where maximum growth (OD_{550nm} = 3.9) was achieved. The enzyme activity increased rapidly as cells increased exponentially where the maximum activity of 0.22 U/mL was achieved after 8h and remained constant till 12 h during the stationary phase of growth. Agarase production was optimized using Plackett-Burman statistical design by measuring enzyme activity as a response and the design was validated using a verification experiment; the activity of the enzyme increased from 0.22 U/mL to 0.29 U/mL. *Pseudoalteromonas* sp. MHS agarase was partially purified and its molecular weight (MW) was determined by SDS-PAGE (15-25 kDa). Agarase showed approximately 94% of its activity at 40 °C. The enzyme stability decreased as the temperature increased; the enzyme could retain about 98, 90, 80, 75, and 60% of its activity at 20, 30, 40, 50, and 60 °C, respectively. Biomass of the red alga *Pterocladia capillacea* proved to be a suitable substrate for agarase production using *Pseudoalteromonas* sp. MHS; the enzyme activity recorded after 24 h of incubation was 0.35 U/mL compared to 0.29 U/mL from the optimized medium.

*Corresponding Author:

Samia S. Abouelkheir,

National Institute of Oceanography and Fisheries (NIOF), Marine Environment Division, Marine Microbiology Laboratory, Egypt;

Email: samiaabouelkhir@yahoo.com

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1. Introduction

Agarases are hydrolytic enzymes found in a wide variety of marine organisms. They're used in biotechnological and commercial operations like decomposing algal polysaccharides, liquefaction of agar and agarose gels, biofilm removal in bioreactors, and the creation of simple sugars, along with in the food sector to make bread, beverages, and low-calorie foods ^[1]. The major component in the cell wall of red algae is agar, which is made up of agarpectin and agarose. To make this complex polysaccharide available for microorganisms, agarases which can hydrolyze agar into oligosaccharides or monosaccharides are necessary ^[2]. Many important qualities of oligosaccharides generated from agar include delayed starch degradation, bacterial growth suppression, anticancer and antioxidant actions, etc. ^[3].

Macroalgae are important primary producers in coastal environments, accounting for a large portion of the biomass and having an ecological role ^[4]. The study of the epiphytic microbiota of algae remains unexplored despite their huge biodiversity. Furthermore, because these microorganisms interact with algae, they're thought

to be a good source of algal-specific enzymes (e.g., carrageenases, agarases, and alginate lyases) ^[5,6].

Ulva represents a vital structural constituent of coastal intertidal environments and acts as a microbial community's starting point. These communities attached to living surfaces, especially those associated with marine macroalgae of the genus *Ulva*, are recognized to harbour a large number of accompanying microorganisms with host-specific colonisation configurations that are influenced by macroalgae features such as cell wall constituents and defence mechanisms ^[7]. The creation of these polysaccharides by macroalgae encourages epiphytic bacteria to produce enzymes that can breakdown a wide range of compounds ^[5]. Comba-González et al., (2016) ^[1] constructed a flowchart illustrating the various investigational procedures intended for recognizing enzymes generated by *Ulva* associated-epiphytic bacteria.

Many bacteria have been found in saltwater and marine sediments that can hydrolyze and metabolise agar as a carbon and energy source, creating agarases, which catalyse the hydrolysis of agar ^[8]. Agarolytic bacteria are classified into two classes based on their ability to digest

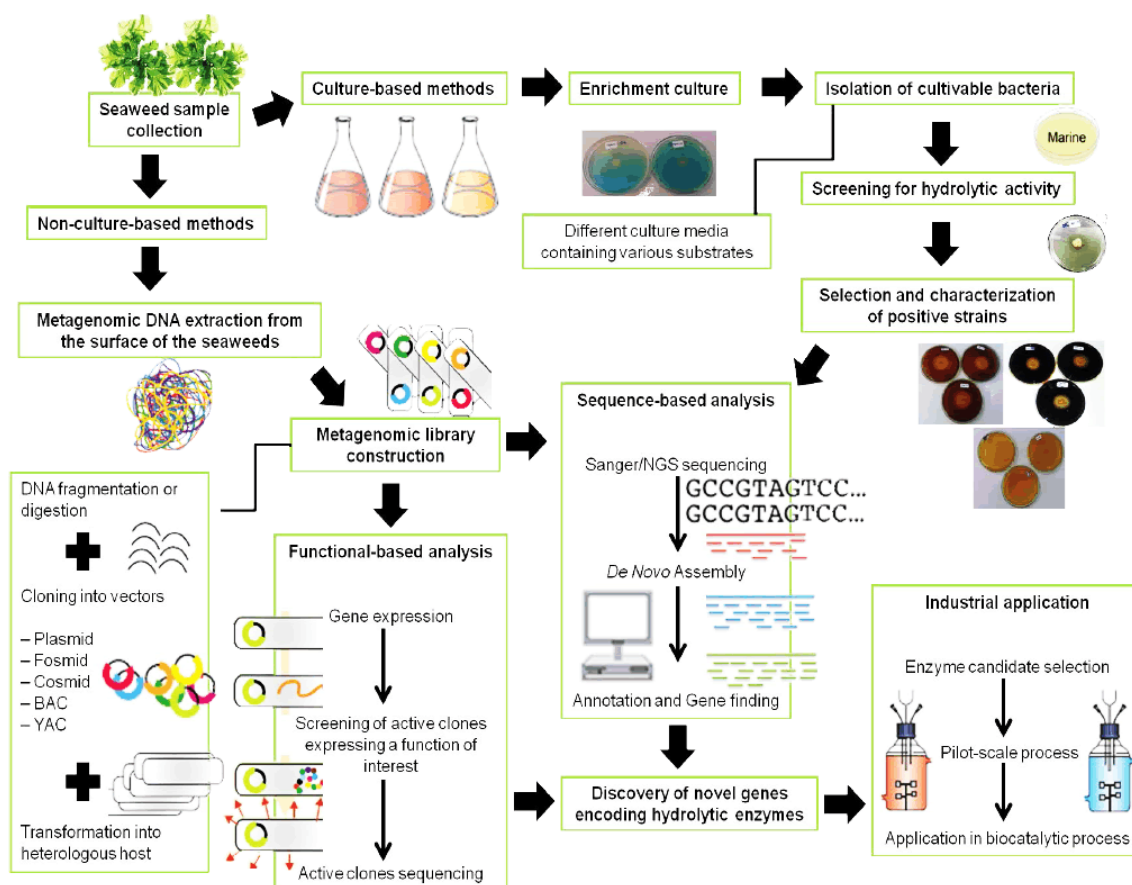


Figure 1. A schematic representation depicting the various experimental approaches used to study macroalgal epiphytic bacteria and their hydrolytic enzymes ^[1].

solid agar. Bacteria in group I soften the agar, causing depressions adjacent to the colonies, while bacteria in group II cause the agar to liquefy extensively^[9]. Because agar is a polysaccharide generated by marine seaweeds, most agarolytic bacteria originally obtained from marine habitats; they include *Pseudomonas* sp.^[10], *Pseudoalteromonas* sp.^[11], and *Micrococcus*^[12]. Only a few strains, such as *Streptomyces coelicolor* A3 (2)^[13] and *Streptomyces lavendulae* UN-8^[14], have been identified from non-marine environments. *Pseudoalteromonas* species are commonly detected in the presence of marine eukaryotes^[15,16]. *Pseudoalteromonas* species have been found to produce a number of enzymes that aid in the degradation of polysaccharides derived from marine algae such as alginate, agar, and carrageenan^[11].

Up to our knowledge, there are just a limited numbers of publications linked to the improvement of agarase production using wild type bacteria that we are aware of^[17-19]. Statistical designs have thus been successfully employed to improve the productivity of several bioprocesses^[18]. The Plackett-Burman design (PB) cuts down on the number of tests while still producing relevant findings. The first stage is to use a fractional factorial design to assess the comparative significance of numerous ingredients within a complex culture media, selecting levels of variables that have substantial effects on enzyme activity. The optimal medium's validity is checked against the basal medium and anti-optimum one as a second step. If *n* factors are to be explored in this study, a complete factorial design would necessitate 2^{*n*} experiments^[18].

Therefore, the target of the current search was to isolate and identify a marine agarolytic bacterium from the epiphytic microbiome of the green macroalga *Ulva lactuca*. It is also aimed to elucidate the factors affecting agarase production employing Plackett-Burman experimental design. In addition, partial purification of the enzyme was considered. Furthermore, biomass of the red seaweed *Pterocladia capillacea* was evaluated as a substrate for agarase production using *Pseudoalteromonas* sp. MHS.

2. Materials & Methods

2.1 Bacterial Strain

Pseudoalteromonas sp. MHS utilized throughout the study was isolated from *Ulva lactuca* surface collected from the Mediterranean Sea at El-Anfoushi district, Alexandria, Egypt, and identified using the 16S rRNA sequence analysis.

2.2 Microbiological Medium

The medium used in this study was nutrient broth (NB) (5 g peptone, 3 g yeast extract) dissolved in 1 L

seawater and supplemented with 0.1% agar. In case of solid medium nutrient agar (NA), 1.5% agar was used for solidification and sterilized using autoclave at 121 °C for 20 min. Initial pH was 7.5.

2.3 Reagents for Reducing Sugar Estimation

Dinitrosalicylic acid (DNS) reagent: 0.2 g phenol and 0.05 g sodium sulfite were dissolved in 100 mL 1% NaOH, and 1 g dinitrosalicylic acid was freshly added to the solution just before use^[20]. Rochelle salt solution: 40 g of KNaC₄H₄O₆·4H₂O were dissolved in 100 mL distilled H₂O. Lugol's Iodine Solution: Lugol's Iodine solution was prepared by dissolving 1 g iodine crystals and 2 g KI in 300 mL distilled H₂O^[21]. Phosphate Buffer Saline (PBS): 20 mM PBS was prepared by dissolving 16 g of NaCl, 0.4 g of KCl, 2.88 g of Na₂HPO₄, and 0.48 g of KH₂PO₄ in distilled water to reach 1000 mL final volume at pH 7.6.

2.4 Solutions for SDS-Polyacrylamide Gel Electrophoresis

Solutions and buffers for SDS-Polyacrylamide gel electrophoresis were prepared according to A Guide to Polyacrylamide Gel Electrophoresis and Detection^[22].

2.5 Sample Collection

The green alga *Ulva lactuca* was collected from the Mediterranean Sea at El-Anfoushi district, Alexandria, Egypt. The red alga *Pterocladia capillacea* was collected from Abu Qir district, Alexandria, Egypt.

2.6 Isolation of Agarolytic Bacteria

The algal thallus *Ulva lactuca* was gently washed and dispensed in a 100 mL Erlenmeyer's flask containing 20 mL sterile seawater and shaken at 150 rpm for 1h to detach the bacteria on the surface. Subsequently, 0.2 mL aliquot from flask was plated on NA medium. Agarolytic activity was determined by agar liquefaction or shallow depressions forming around the colonies after 48 hours of incubation at 25 °C. One isolate designated as MHS showing clearance zone and softening of agar around the colonies was chosen for further investigations. To confirm agarolytic activities, NA plates were inoculated with MHS and incubated at 25 °C for 2 days before being stained with Lugol's iodine solution to detect the existence of a clear zone around the colonies^[23-26].

2.7 Identification and Characterization of the Selected Isolate

2.7.1 Phenotypic Characterization

Negative Stain, Capsule Stain, and KOH tests were performed according to Moyes et al., (2009)^[27], Duguid,

(1951)^[28], and Buck, (1982)^[29], respectively. Screening for polyhydroxyalkanoate (PHA) accumulation was done by two methods; Plate assay method and slide staining method according to Phanse et al., (2011)^[30].

2.7.2 Genotypic Characterization

Genomic DNA was extracted from the pure selected isolate and then analysed by electrophoresis according to the method described by Sambrook et al., (1989)^[31]. The 16S *rRNA* gene was amplified by polymerase chain reaction (PCR) using the primer pair 8F:5'-AGAGTTTGATCCTGGCTCAG-3' 1525R: 5'-AAGGAGGTGWTCCARCC-3'. The PCR reaction and product was sequenced using the sequencing facility provided by the U.S.B. American Company via SIGMA-Egypt.

2.8 Factors Affecting Growth of *Pseudoalteromonas* sp. MHS

The effect of temperature was examined by inoculating NB flasks adjusted at pH 7.5 with *Pseudoalteromonas* sp. MHS from previously prepared seed culture ($OD_{550nm} \approx 1 \pm 0.1$) and incubated at diverse temperatures (5, 10, 15, 20, 25, 30 and 37 °C). The cultures were checked for growth after 24 h. Similarly, NB flasks were prepared with different seawater concentrations (0, 25, 75, and 100%); the pH was adjusted at 7.5 and incubated with the same condition to study the seawater requirement.

2.9 Agarase Production in Relation to Bacterial Growth

A standard inoculum (2%) from previously prepared seed culture ($OD_{550nm} \approx 1 \pm 0.1$) was used to inoculate flasks each containing 20 mL of NB medium supplemented with 0.1% agar and adjusted at pH 7.5. Flasks were incubated at 120 rpm and 25 °C on a rotary shaker incubator (Scilogex SK-O330-Pro, USA). Bacterial growth and enzyme activity were evaluated at time intervals. Bacterial growth was expressed as OD 550 nm using a spectrophotometer (Optima SP-300, Japan).

2.10 Crude Enzyme Preparation, Semi Quantitative and Quantitative Activity Assay

The crude enzyme was obtained by centrifugation of bacterial culture at 10,000 x g for 10 min in microfuge (Hettich® MIKRO 120, Germany). The culture filtrate obtained after cell removal was considered the crude enzyme. A semi-quantitative assay was used to screen for agarase activity, based on the size of clearance zone of the cell free supernatant of *Pseudoalteromonas* sp. MHS. The experiment was carried out in a medium containing

only seawater amended with 15 g/L agar. The wells on agar plates were filled with 10 µl of cell-free supernatant, and the plates were incubated for 4 hours at 30 degrees Celsius. The formation of clear zones was confirmed by the addition of iodine solution. A pale-yellow zone around colonies was detected against a brown-violet background, indicating agar deterioration^[23,32]. The increase in the concentration of reducing sugars was determined spectrophotometrically using the 3, 5-dinitrosalicylic acid (DNS) technique to determine agarase activity^[33]. Under the assay conditions, one unit (U) of agarase is defined as the quantity of enzyme that produces 1 µmol of galactose per minute^[34]. Galactose was employed as a reference reducing sugar for preparing a calibration curve.

2.11 Optimizing Culture Conditions for Agarase Production Using Plackett- Burman Design

Enhancement of some physical and nutritional factors influencing agarase production was performed using Plackett-Burman experimental design. This fractional factorial design^[35] was used in this research to reflect the relative significance of several variables on agarase production by *Pseudoalteromonas* sp. MHS. In this study, seven independent variables were screened in nine different combinations according to the PB design matrix's eight runs. The selected variables included yeast, peptone, agar, pH, agitation, inoculum size, and seawater concentration. A high (+) and low (-) level was evaluated for each variable. The response was estimated using agarase activity. The following equation was used to find the main effect of each variable:

$$Ex_i = (\Sigma\pi_i^+ - \Sigma\pi_i^-)/N$$

Where Ex_i is the variable main effect, $\Sigma\pi_i^+$ and $\Sigma\pi_i^-$ are responses in trials where the independent variable (X_i) was present in high and low concentrations, respectively, and N is the number of trials divided by two. Accordingly, a main effect chart with a positive and negative signs show that the high and low concentrations, respectively, of this variable was closer to optimum. The factor that had no effect would give a value of zero if no interactions existed. To determine the variable significance, statistical t-values for equal unpaired samples were calculated using Microsoft Excel.

2.12 Partial Purification of *Pseudoalteromonas* sp. MHS Agarase Enzyme

400 mL of a 12 h old culture grown under optimized conditions were centrifuged at 10,000 x g for 10 min in a cooling centrifuge (HERMLE Z 36 HK, Germany) at 4 °C to remove the bacterial cells. The enzyme in the

supernatant was precipitated by adding 75 % saturation solid ammonium sulphate and stirring slowly for 1 hour. At 4 degrees Celsius, the solution was left overnight. Centrifugation at 12,000 x g for 15 minutes in a cooling centrifuge at 4 °C pelleted the precipitated protein that resuspended in 20 mL PBS and dialyzed against the same buffer in a dialysis bag in a refrigerator with intermittent change of buffer every 4 h for 1 day. The dialyzed sample was considered as partially purified agarase^[11].

2.13 Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) of Protein

The SDS-Polyacrylamide gel electrophoresis of protein was performed according to Sambrook et al., (1989)^[31]. The broad range molecular weight markers “Promega” consisting of nine clearly identifiable bands (10, 15, 25, 35, 50, 75, 100, 150, and 225 kDa) were used in the molecular weight determination of the protein.

2.14 Influence of Temperature on the Activity and Stability of the Partially Purified Agarase

The optimum temperature for the activity of the purified agarase was determined in PBS at different temperatures from 20 °C to 60 °C under the assay conditions. In this experiment, the partially purified agarase was mixed with substrate and incubated at several temperatures. The DNS method was used to determine agarase activity. The thermostability of agarase was determined via measuring the enzyme’s residual activity after a 30-minute pre-incubation period at temperatures ranging from 20 to 60 degrees Celsius^[36,37].

2.15 Agarase Production Using Red Seaweed Biomass by *Pseudoalteromonas* sp. MHS

The red seaweed *Pterocladia capillacea* was collected from the Mediterranean Sea at Abu Qir, Alexandria, Egypt, dried at 60 °C overnight, and ground using a porcelain mortar and pestle. A 500 µm sieve was used to attain particle size homogeneity in the final samples. 800 µl of the seed culture were inoculated in 20 mL Erlenmeyer flasks containing sterile seawater supplemented with different concentrations of the red seaweed (5, 10, 15, 20 g/L), adjusted at pH 9, and incubated at 25 °C under shaking condition (230 rpm). The agarase activity was measured for each flask at 12 and 24 h.

2.16 Statistical Data Analysis

All investigations were done in duplicates. The results were statistically analysed and accomplished by using Microsoft Excel. The data were expressed by means ±

SE and ± SD. The significant values were determined at P-value < 0.05.

3. Results & Discussion

An attempt was carried out to isolate and identify of a local marine agarolytic bacteria associated with the surface of the green alga *Ulva lactuca*. Isolate MHS recovered on a nutrient agar plate (Figure 2a) showed clear zone and softening of the agar around the colonies (Figure 2b) after 2 days of incubation at 25 °C. Flooding the plates with Iodine caused the presence of pale-yellow zones around colonies in contrast to a brown-violet background (Figure 2c), which was considered a confirmation of agar-degrading activity^[23,9,24].



Figure 2. MHS white mucoid colonies on NA plates after 48 h of incubation at 25 °C (a); Clear zone formation and depression around the colony (b); Yellow zone against brown background formed after flooding with Lugol’s iodine solution (c).

MHS cells appeared as rods under the microscope when stained by the negative stain, and appeared to be encapsulated when stained by the capsule stain (Figure 3a, 3b). The dark blue colour of colonies after flooding the plate with Sudan Black B was taken as positive for accumulation of PHA as a storage material^[30] and was further confirmed by Sudan black staining of cells as dark blue spots appeared inside red cells (Figure 3c). KOH test was used as an alternative to Gram stain; a positive result indicated that the isolate is Gram negative.

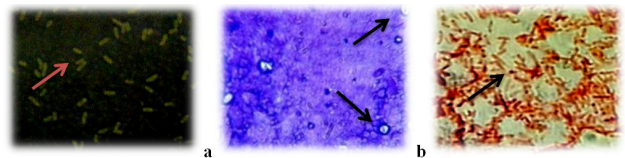


Figure 3. Light micrograph of MHS: Negative stain showing rod shape cells (red arrow) (a); Capsule stain, black arrows: colourless bacterial capsules with stained cells against dark background, Magnification×1,000. Micrograph of MHS showing the PHA granules produced in the form of dark granules in the bacterial cells (c)

Many bacterial taxa having agarolytic activity have been discovered, primarily in saltwater and marine sediment^[38,25] and many reports have been made regarding

isolating agarolytic bacteria from seaweeds [39,40,34]. In a previous study, Furusawa et al. (2017) [26] stated the isolation of the agarolytic bacterium *Persicobacter* sp. CCBQB2 from *Ulva* sp. collected from a Malaysian coastal location.

In a sequential step, it was necessary to identify isolate MHS by amplifying the gene coding for the 16S rRNA using the specific primer pair mentioned in the materials and method section. The amplified fragments (1500 bps) were sequenced by the U.S.B. American Company's sequencing laboratory, SIGMA-Egypt. 16S rRNA gene sequence of MHS displayed 99% resemblance to several sequences of *Pseudoalteromonas* spp. The 16S rRNA gene sequence was deposited as KT275859 in GenBank and classified as a member of the genus *Pseudoalteromonas*, family Pseudoalteromonadaceae, order Alteromonadales, class, γ -Proteobacteria, phylum Proteobacteria and was designated as *Pseudoalteromonas* sp. MHS. The phylogenetic bond of the amplified 16S rRNA sequence and its nearby relatives was analysed using the services provided by the Ribosomal Database Project (phylogenyfr.com) and the results are summarized in the dendrogram in Figure 4.

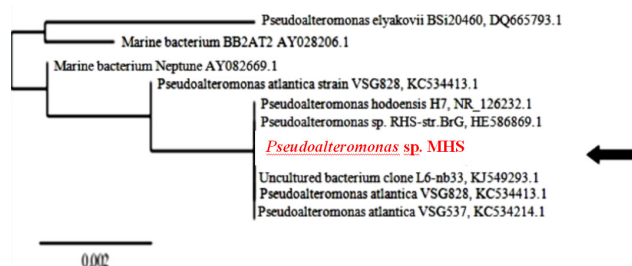


Figure 4. Shows the position of strain MHS among other closely related *Pseudoalteromonas* species in a neighboring phylogenetic dendrogram based on 16S rRNA

Pseudoalteromonas (*P.*) was previously isolated from the green alga *Ulva*; Egan et al. (2001) [41] isolated two strains, *Pseudoalteromonas ulvae* UL12T and UL13 from the surface of *U. lactuca*, collected from the rocky intertidal zone near Sydney, on the east coast of Australia. *P. prydzensis* alex and *P. sp. alex* were also isolated from Mediterranean saltwater rocks and sponge in Alexandria, Egypt [15,16]. In addition, the agar-hydrolysing bacterium *P. hodoensis* H7 was isolated from a coastal seawater sample obtained from Ho Island in the West Sea, South Korea [11].

Pseudoalteromonas sp. MHS could grow at temperature range 5-30 °C with maximal cell growth at 30 °C while no growth was noticed at 37 °C (Figure 5a). This indicates that *Pseudoalteromonas* sp. MHS favours the lower temperature than the higher one. Similar to the other agarase producing marine bacterium [19,11], the strain is considered a psychrotroph. Sodium chloride was

also required for MHS growth. It grew well at different concentrations of seawater but not in the absence of seawater (Figure 5b). The obtained data refer to the halophilism of the isolate and its classification as a marine bacterium.

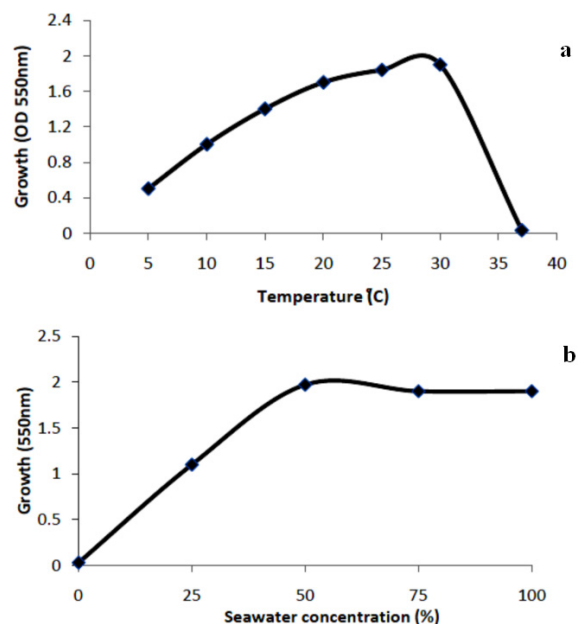


Figure 5. Growth of MHS on NB for 24 h at different temperatures (a), and with different concentrations of seawater at 25 °C (b)

3.1 Agarase Activity in Relation to Growth

Agar degradation in relation to bacterial cell growth was studied during batch fermentation in NB supplemented with 0.1% agar under shaken condition at 25 °C. Figure 6 clarifies that growth and enzyme production were parallel. Cells demonstrated a lag phase of 2 h, after that an exponential growth which prolonged till 10h where maximum growth ($OD_{550nm} = 3.9$) was achieved. The enzyme activity increased rapidly as cells increased exponentially where maximum activity of 0.22 U/mL was achieved after 8h and remained constant till 12h during the stationary phase of growth. Based on semi quantitative assay i.e. measurement of inhibition zones formed, a zone of 16 mm was recorded just after inoculation with seed culture, and increased rapidly from 18 to 20 mm at 4-6 h reaching a maximum clearance zone of 21 mm after 8h of incubation then remained constant till 12 h of incubation (Figure 6a, 6b). The data obtained is in agreement with the previous work of Faturrahman et al. (2011) [23], and Chi et al. (2014) [11].

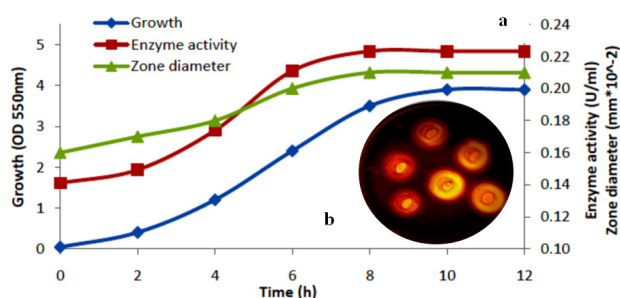


Figure 6. Growth and agarase activity of *Pseudoalteromonas* sp. MHS incubated at 25 °C and 120 rpm at different time intervals (a), Clearance zones resulting from the inoculation of 10 μ l of cell-free supernatant of *P.* sp. MHS on agar plate (15 g/L agar in SW) and incubated at 30 °C for 4 h followed by flooding the plate with Lugol's iodine solution (b).

3.2 Optimizing Culture Conditions for Agarase Production Using Plackett-Burman Design

The PB design was utilised to screen the significant factors relevant for agarase production by *Pseudoalteromonas* sp. MHS grown on NB medium supplemented with agar. These factors were yeast, peptone, agar, pH, agitation, inoculum size, and seawater concentration. In this experiment 7 variables were evaluated by 9 experiments and the levels of each variable were determined. The application of statistical design was performed in a 'two-phase' optimization process. The initial step was to look for significant influences on agarase production followed by verification experiment to validate the results under precise, optimised experimental settings. All of the experiments followed a design matrix (Table 1) that was based on the number of variables to be investigated. Each column of the matrix represented an independent variable whose level was varied, and each

row represented a trial. Each variable was evaluated at two levels, a high (+) and a low (-) level^[18]. Agarase activity was measured using the DNS assay after 12 h of incubation.

Table 1. Levels of independent factors in the Plackett-Burman design

Variable	-1	0	+1
Yeast (g/L)	1	3	5
Peptone (g/L)	3	5	7
Agar (g/L)	0.5	1	2
Inoculum size (%)	1	2	4
pH	6	7.5	9
Seawater (%)	50	100	100
Agitation	Static	120 rpm	230 rpm

The main effect of each variable upon enzyme activity was valued and presented graphically in Figure 7. As clearly presented from this chart, variables such as pH, agitation, inoculum size, agar, and yeast concentration had positive effects on agarase production while peptone and seawater concentration had negative effects. Therefore, decreasing the peptone and seawater, and simultaneously increasing pH, shaking, inoculum size, agar and yeast can boost productivity of agarase in the culture medium. In agreement with our findings, Fu et al. (2009)^[17] used a nine-factor, twelve-run Plackett-Burman design to maximise agarase production by *Agarivorans albus* YKW-34 who stated that, the initial pH had a substantial impact on agarase production ($p < 0.05$). Furthermore, in the Plackett-Burman design, yeast, agar, and starting pH exhibited beneficial impacts (t -value > 0) across the range of examined levels.

Table 2. Plackett-Burman design matrix representing the coded values for 7 independent factors, 0 represents the original concentration, -1 denotes the low level and +1 represents the high level for each component. Enzyme activity was calculated as the response.

Trial no.	Yeast	Peptone	Agar	Seawater	Inoculum size	Agitation	pH	Enzyme activity (U/mL)
1	-1	-1	-1	1	1	1	-1	0.19
2	-1	-1	1	1	-1	-1	1	0.18
3	-1	1	-1	-1	1	-1	1	0.2
4	-1	1	1	-1	-1	1	-1	0.186
5	1	-1	-1	-1	-1	1	1	0.238
6	1	-1	1	-1	1	-1	-1	0.19
7	1	1	-1	1	-1	-1	-1	0.14
8	1	1	1	1	1	1	1	0.24
9	0	0	0	0	0	0	0	0.22

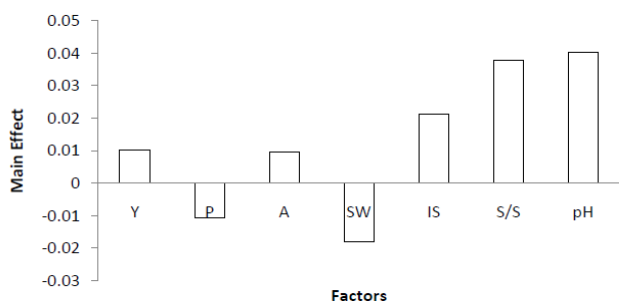


Figure 7. Main effect of the culture factors that affect agarase production by *Pseudoalteromonas* sp. MHS

The statistical significance of the measured response and evaluated main effects of each variable were determined by statistical analysis of the Plackett-Burman experiment using the t-test provided by Excel Microsoft Office (Table 3).

Table 3. Statistical analysis of the Plackett-Burman experimental results

Factors	Significance level (%)	P-value	t-value
Yeast extract	63	0.36185	0.388315
Peptone	65	0.348448	-0.41274
Agar	63	0.362537	0.372091
Seawater	74	0.252522	-0.71774
Inoculum size	78	0.216116	0.853751
Agitation	93	0.062652	1.780418
pH	95	0.04832	1.94318

In the present study, confidence levels of 95% and 93% were reported for pH and agitation respectively. On the other hand, confidence levels lower than 90% were found for inoculum size and seawater concentration of 78% and 74% respectively. Yeast extract, agar and peptone concentrations had confidence levels of 63%, 63% and 65% respectively. The contribution of each factor is represented in the following Pareto chart (Figure 8).

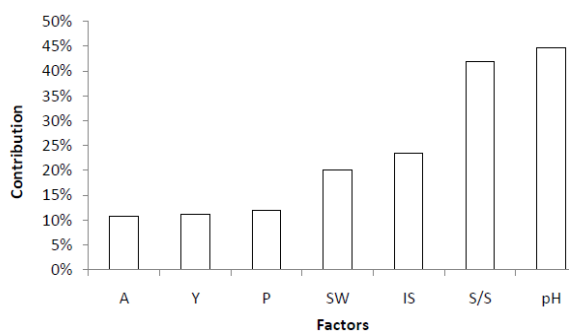


Figure 8. Pareto chart of standardized effects for agarase from *Pseudoalteromonas* sp. MHS after 12 h of cultivation for the Plackett-Burman design.

Verification experiment

To forecast the near-optimal levels of independent variables, a verification experiment was carried out in triplicate. The values of an anti-optimized medium were the polar opposites of those of an optimized one. As we notice from Figure 9, the optimized medium was the preferable medium for agarase production by *Pseudoalteromonas* sp. MHS showing the highest enzyme activity (0.29 U/mL) when cultivated in optimized medium and the lowest (0.14 U/mL) when cultivated in anti-optimized medium while growing in the basal medium showed corresponding agarase activity of 0.22 U/mL.

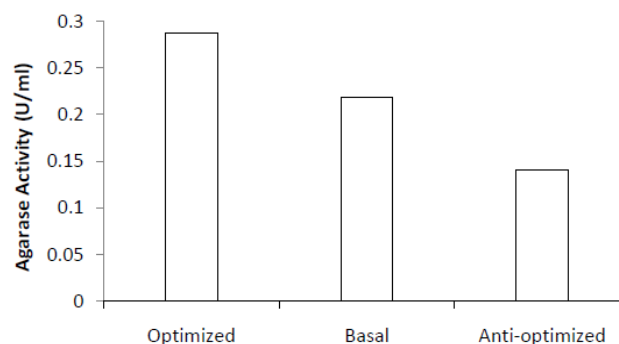


Figure 9. Verification experiment of the applied Plackett-Burman statistical design, comparing agarase activity produced by *P.* sp. MHS grown on optimized, basal, and anti-optimized media for 12 h at 25 °C.

3.3 Purification and Characterization of *P. sp. MHS* Agarase

3.3.1 Enzyme Purification

Partial purification of the bacterial crude extract was achieved after salting-out with ammonium sulfate followed by enzyme dialysis. Many literature have reported the use of ammonium sulfate in the purification of agarase^[24,42,43].

3.3.2 SDS-PAGE of the Enzyme

The partially purified enzyme was subjected to SDS-PAGE that confirmed only one band was observed. The molecular weight (MW) of the protein band was between 15 kDa and 25 kDa (Figure 10).

In accordance to the obtained molecular weight (between 15 kDa and 25 kDa) of the protein band in this work, the reported MW of agarase differs from values as low as 20 kDa for *Bacillus subtilis*^[9], to as high as 80 kDa, in the case of *Pseudomonas*-like bacteria^[36].

Saraswathi et al. (2011)^[9] reported that the molecular weight of purified agarase from *Bacillus subtilis* (20 kDa) was close to those reported for agarases, and in contrast to that produced by *Pseudoalteromonas hodoensis* H7, 35 kDa^[11] and *Pseudoalteromonas* sp. NJ21, 80 kDa^[36].

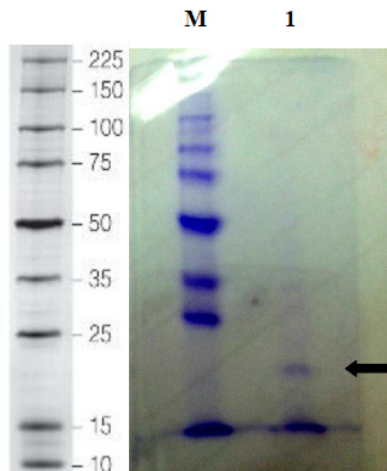


Figure 10. SDS-PAGE analysis of partially purified agarase; Lane M, molecular weight standards; Lane 1 partially purified agarase after $(\text{NH}_4)_2\text{SO}_4$ precipitation

3.3.3 Effect of Temperature on Agarase Activity and Stability

Temperature is thought to be a crucial factor in enzyme activity. Therefore, temperature effects on *P. sp.* MHS agarase activity were investigated by assessing the activity at various temperatures (20 °C-60 °C). As shown in Figure 11a, agarase had a peak temperature of 50 °C and approximately 94% of the supreme activity was detected at 40 °C. When the temperature reached 60 °C, the enzyme had only 87% of its optimal activity whereas, 54% and 82% of the optimal activity were noticed at 20 and 30 °C, respectively. The effect of temperature on the stability of the purified enzyme was studied in the range of 20 °C-60 °C. As noticed from Figure 11b, the stability of the enzyme decreased as the temperature increased; the enzyme could retain about 98%, 90%, 80%, 75%, and 60% of its activity at 20, 30, 40, 50, and 60 °C, respectively.

Because dense bundles of gelled agar inhibit enzyme action, the temperature optima of various agarases are greater than the agar gelling temperature^[24]. The optimum temperature of 50 °C for *Pseudoalteromonas* sp. MHS agarase is similar to that of agarase-b produced by *Agarivorans albus* OAY02^[34], less than that of 60 °C for *Catenovulum agarivorans* YM01T agarase^[25], and higher than that of 30 °C for *Pseudoalteromonas* sp. NJ21 agarases^[36], 35 °C of *Agarivorans* sp. JA-1 agarase^[44],

40 °C of agarase-a from *Agarivorans albus* OAY02^[34], and 45 °C of *Pseudoalteromonas hodoensis* H7 agarase^[11]. The increased thermostability of *Pseudoalteromonas* sp. MHS will be beneficial for industrial applications.

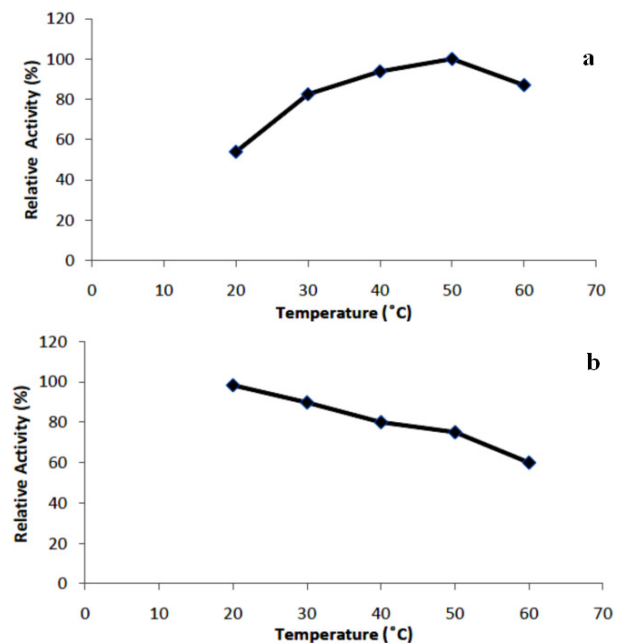


Figure 11. Effect of different temperatures on agarase activity (a) and stability (b)

3.4 The Use of Red Seaweed *Pterocladia capillacea* as Substrate for Agarase Production

While chemical degradation of biomass is a faster and less expensive choice, it has a number of drawbacks, including the production of toxic waste that is harmful to the environment and toxic end products that are not fermentable. Developing an appropriate enzymatic method for biomass degradation is becoming increasingly crucial in this regard. The isolation of microorganisms with strong biopolymer hydrolyzing activity is critical for the successful development of marine biomass for industrial use. Due to its employment as an industrial resource and a depolluting plant for cleaning inland sea areas and eutrophied seawater, the amount of seaweed trash is steadily increasing^[45]. As a result, the recycling of organic substances and the protection of the marine environment needs the utilisation of seaweed waste^[46,47].

Therefore, in this experiment, it was aimed to produce agarase from a low cost substrate for economic purposes. The red seaweed *Pterocladia capillacea* was used as a natural medium with no additives for the growth of *Pseudoalteromonas* sp. MHS. Figure 12a illustrates the medium prepared with the 20 g/L *Pterocladia capillacea* in seawater after autoclaving while Figure 12b illustrates

the liquefaction of algal biomass after inoculation by *Pseudoalteromonas* sp. MHS and incubation at 25 °C and 230 rpm for 24 h.

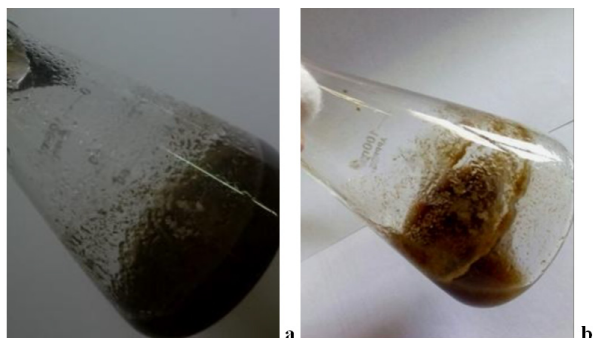


Figure 12. Twenty g/L *Pterocladia capillacea* in seawater after autoclaving (a), and medium liquefaction by *Pseudoalteromonas* sp. MHS grown at 25 °C and 230 rpm for 24 h (b)

Data in Table 4 depict that an activity of 0.27 U/mL of agarase was obtained after 12 h of incubation for the three different concentrations of the seaweed used. However, the flask containing the highest concentration of the seaweed (20 g/L) was only partially liquefied by the bacterium and the enzyme activity couldn't be measured due to its high viscosity. Consequently, measurements taken after 24 h of incubation were higher compared to those taken after 12 h.

Table 4. Agarase activity of *P. sp.* MHS grown on red seaweed

Seaweed conc. (g/L)	Incubation Time (h)	
	12	24
	Enzyme activity (U/mL)	
5	0.27	0.26
10	0.27	0.3
15	0.27	0.31
20	*Nd	0.35

*Nd: Not determined

It is worth to mention that the agarase activities recorded after 24 h of incubation (0.3, 0.31, and 0.35 U/mL) using 10, 15, and 20 g/L of biomass, respectively were higher than that of 0.29 U/mL obtained from the optimized medium of PB design. This indicates that red seaweeds could replace media used for agarase production and are suitable sources for bacterial nutrition.

According to the findings of this study, red algae of the genus *Pterocladia* are often found in the seas of Lebanon, Egypt, Brazil, Italy, and other countries for the industrial production of gelling galactans^[48,49]. Red seaweed has a carbohydrate composition of 30%-60%,

with agar and carrageenan accounting for the majority of it. Unused seaweed trash is commonly disposed of via landfill, incineration, or dumping into the sea, all of which pollute the environment. Kang & Kim (2015)^[46] isolated a *Bacillus* sp. SYR4 strain that was able to use seaweed waste as a carbon source by degrading both agar and carrageenan and producing reducing sugars, which served as a substrate for bioethanol production, yielding 7-10 wt% ethanol^[50].

4. Conclusions and Future Perspectives

This study provides evidence that marine bacteria, particularly those associated with algae contribute in the production of hydrolytic enzymes such as agarase. The study evaluated a strain of *Pseudoalteromonas* sp. MHS isolated from the green alga *Ulva lactuca* to produce agarase and degrade agar. Agarase activity was optimized via an experimental Plackett-Burman design. The utilization of red algae that usually disposed in the environment as substrate for agarase production was examined for an economic purpose. Surprisingly the activity obtained was higher than that recorded with microbiological media. We therefore recommend the use of the huge amount of seaweeds disposed in the environment as substrate for microbial production of enzymes. Although agarases have an old history of application in various industries, they are still in the experimental stages and are not commercially available. Therefore, they are expected to increase further study in the very near future thereby leading to further advancement in the biotechnology fields.

Author Contributions

M.M.S. conceived and conducted the experiments; S.S.A. conceived the idea, analyzed and interpreted the data, wrote the manuscript; M.E.M., H.A.G., and S.A.S. conceived the research idea, analyzed the data, and edited the manuscript.

Competing Interests

The authors declare no competing interests.

Data Availability Statement

Correspondence and requests for materials should be addressed to S.S.A. The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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