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Bacillus sp. R2 Agarase Production: Medium Composition and Fermentation Conditions Optimization

Ben Amar Cheba * ID
Biology Department, College of Science, Jouf University, P.O. Box: 2014, Sakaka, Saudi Arabia.
***Correspondence:** omacheb@gmail.com; Tel.: +966-536-832-385

ORCID ID: 0000-0002-8370-0445

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Abstract: Agar, which can be degraded by agarase, is composed of agarose and agaropectin. Agarases producing microorganisms have been isolated from many sources, including seawater, marine sediments, marine algae, marine mollusks, fresh water, and soil. The present study was firstly planned to investigate the effect of medium composition and culture conditions on agarase production by *Bacillus* sp. R2 in shake flasks (submerged fermentation), using one factor at time technique (OFAT). The highest agarase activity was obtained in 0.2% agar as essential carbon source, 0.2% lactose as secondary carbon source, 0.5% NH_4Cl as nitrogen source, 5% NaCl and 5% culture seed as best inoculum size. The production also was optimum at initial pH: 7, temperature 30 °C, and 24 h incubation period at 150 rpm shaking. The agarase hyperactivity in this cost-effective economic medium composition and culture condition enlighten the potential application of *Bacillus* sp. R2 as promising new source for agarase production.

Keywords: *Bacillus* sp. R2; agarase; submerged fermentation; production; optimization.

1. Introduction

Agar is a hydrophilic, phycocolloid which forms the major component of red macroalgae cell wall, especially the genera of *Gracilaria* and *Gelidium* [1]. Furthermore, agar is a complex polysaccharide made up of agarose and agaropectin [2].

Agarases are members of glycoside hydrolases, which catalyze the hydrolysis of agar or agarose into oligosaccharides. Agarases were classified according to their cleavage pattern into α-agarase (E.C. 3.2.1.158) and β-agarases (EC.3.2.1.81), which produce agarooligosaccharides and neoagarooligosacchrides via cleaving α-(1→3) and β-(1→4) glycosidic bonds, respectively [2]. Oligosaccharides extracted by agarases have remarkable bioactivities which enable them for wide biotechnological applications ranging from biofuel and food to pharmaceutical and medical fields [3-6]. During the last decades, several microbial agarases have been isolated from different fungal and bacterial genera [7-9]. However, few reports were recorded about *Bacillus* agarase in general and very scare literature about marine *Bacillus* agarase [10,11]. For this reason, we focused in this investigation on marine *Bacillus* agarase production optimization.

In our previous studies, *Bacillus* sp. R2 chitinase and α-amylase as well as *Vibrio* sp. R1 agarase were optimized [12-16]. The present work aimed to studying the effect of carbon and nitrogen sources as well as fermentation conditions on *Bacillus* sp. R2 agarase production which will be determined using one variable at time technique (OVAT) in shake flasks submerged fermentations.

2. Materials and Methods

2.1. Chemicals and Bacterial Strain: Galactose, agarose, lactose, bovine serum albumin (BSA), Sodium alginate (Sigma, USA), agar, peptone, tryptone, and yeast extract (Oxoid Hampshire, England). Arabic gum (Janssen Chemica, Belgium), apple pectin (Fison, Germany), 2-Hydroxy 3,5-dinitrosalselic acid (DNSA) (Merck, Darmstadt, Germany). All other chemicals used were of the

highest grade available and were obtained from commercial sources. The strain *Bacillus* sp. R2 used in this study was originally isolated from red sea water, Hurghada, Egypt. It was identified using 16S rRNA gene sequence (GenBank accession number: EU084496) [15].

2.2. Agarase Production Optimization: The factors influencing agarase production were determined using one variable at time technique (OVAT) in shake flasks (submerged fermentation) [17]. The experiments were conducted in 250 ml Erlenmeyer shake flasks containing 50 ml production medium. After sterilization by autoclaving, the flasks were cooled, inoculated with culture seed and maintained under various operational conditions separately. The tested factors were divided as follow:

2.2.1. Effect of Substrate Concentration, Carbon and Nitrogen Sources: Agar initial concentration (0.1–0.5% w/v), carbon sources (glycerol, galactose, lactose, sucrose, starch, dextran, cellulose, pectin, Arabic gum, and alginate), different inorganic (ammonium chloride, ammonium sulphate, and sodium nitrate), and organic (urea, peptone, tryptone and yeast extract) nitrogen sources were tested at a concentration of 0.2% in sea water medium.

2.2.2. Effects of Fermentation Conditions: The tested parameters were sea water strength (25, 50, 75, and 100% v/v), salinity (2.5, 5, 7.5, and 10% NaCl), inoculum size (2.5, 5, 7.5 and 10%), pH (5 to 10), temperature (20, 30, 37 °C), agitation rate (0 to 200 rpm/min).

2.3. Analytical Methods:

2.3.1. Growth Monitoring and Protein Assay: Bacterial cell growth was monitored spectrophotometrically by measuring the absorbance of the cultures at 600 nm as described in Bradford method [18]. Soluble proteins were quantified using bovine serum albumin as calibration standard [18].

2.3.2. Agarase Assay: According to the method of Miller [19], agarase activity was analyzed by estimating the released reducing ends of sugar using D-galactose as standard. One unit of agarase activity was defined as the amount of enzyme required to liberate 1 μmol reducing sugar per min during these conditions.

3. Results and Discussion

3.1. Effect of Medium Composition on Agarase Production:

3.1.1. Effect of Agar Concentration: It is known that an ideal substrate concentration in any fermentation process results in a higher conversion efficiency and an optimum substrate utilization [20]. According to the results illustrated in Fig. (1), agarase production was remarkably affected by agar concentration. The optimum concentration for agarase production was 0.2%. Similar results obtained by Fu *et al.* [21], whose found 0.23% agar for agarase of *Agarivorans albus* YKW-34. Whereas Roseline and Sachindra [22] found 0.5 was the optimum agar concentration for *Acinetobacter junii* PS12B agarase. In general, the optimal agar concentration ranged between 0.1 and 0.5 % as reported by many investigators [20,21,22].

3.1.2. Effect of Carbon Sources: The results presented in Fig. (2) indicated that lactose was the most effective secondary carbon source for agarase production by *Bacillus* sp. R2, while the addition of galactose, significantly decreased agarase production to more than 90% of the maximum. These findings confirmed the catabolite repressive effect of galactose on agarase production. Similar observation was reported by Fu *et al.* [21], whose found 50% of agarase production reduction to the maximum of *Agarivorans albus* YKW-34, when agar was combined with monosaccharides (glucose and galactose). In contrast, Choi *et al.* [23] showed that the supplementation of ASW-YP medium with 0.4% glucose give the highest agarase activity for *Micrococcus* sp. GNUM-08124.

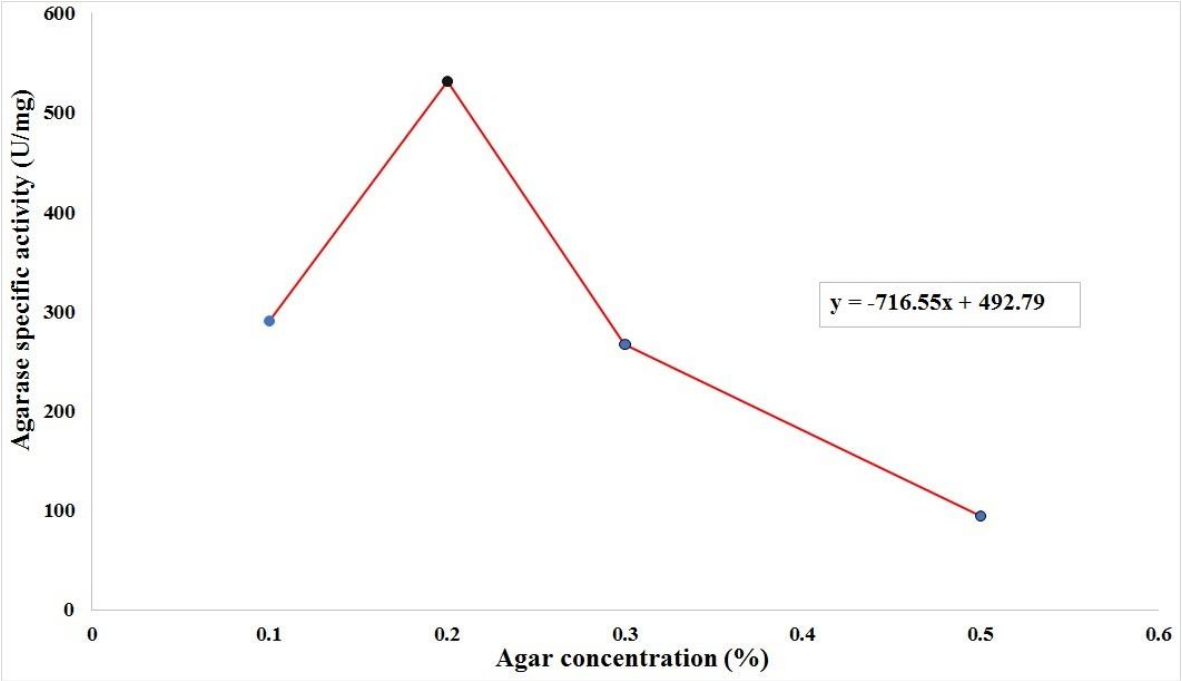

Fig. 1: Effect of agar concentration on agarase production.

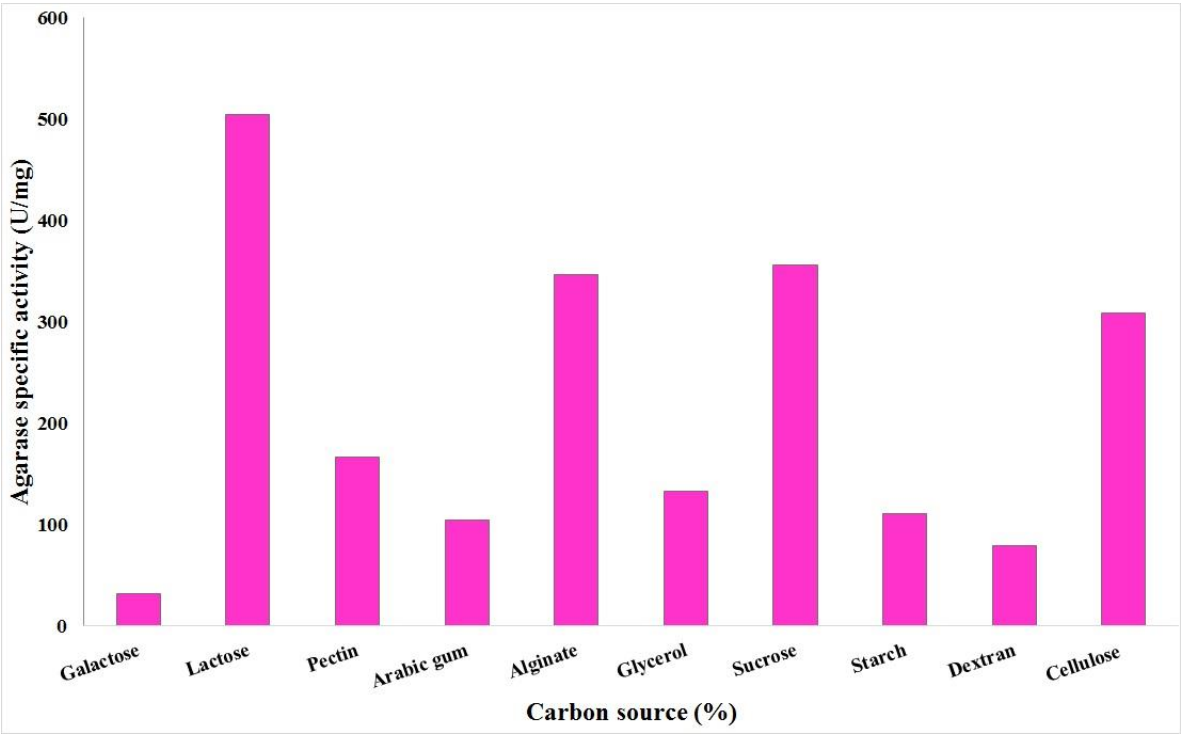

Fig. 2: Effect of carbon sources on agarase production.

3.1.3. Effect of Nitrogen Sources: Various organic and inorganic nitrogen sources supplementations were tested for enhancing agarase production (Fig. 3). The results indicated the suitability of inorganic nitrogen sources, and NH_4Cl was the best source yielding the maximum agarase specific activity. Similar results were presented for *Acinetobacter* sp. AGLSL-1 [24], *Acinetobacter junii* PS12B [22], and *Cytophaga flevensis* [17]. The agarase production was optimal with inorganic nitrogen sources (ammonium nitrate and sodium nitrate) for both the above-mentioned species. In contrast, yeast extract was the most preferable for agarase production by *Agarivorans albus*

YKW-34 [21], and *Pseudomonas aeruginosa* AG LSL-11 [25], respectively. Generally, it seemed that the preference for nitrogen source varies with microorganisms.

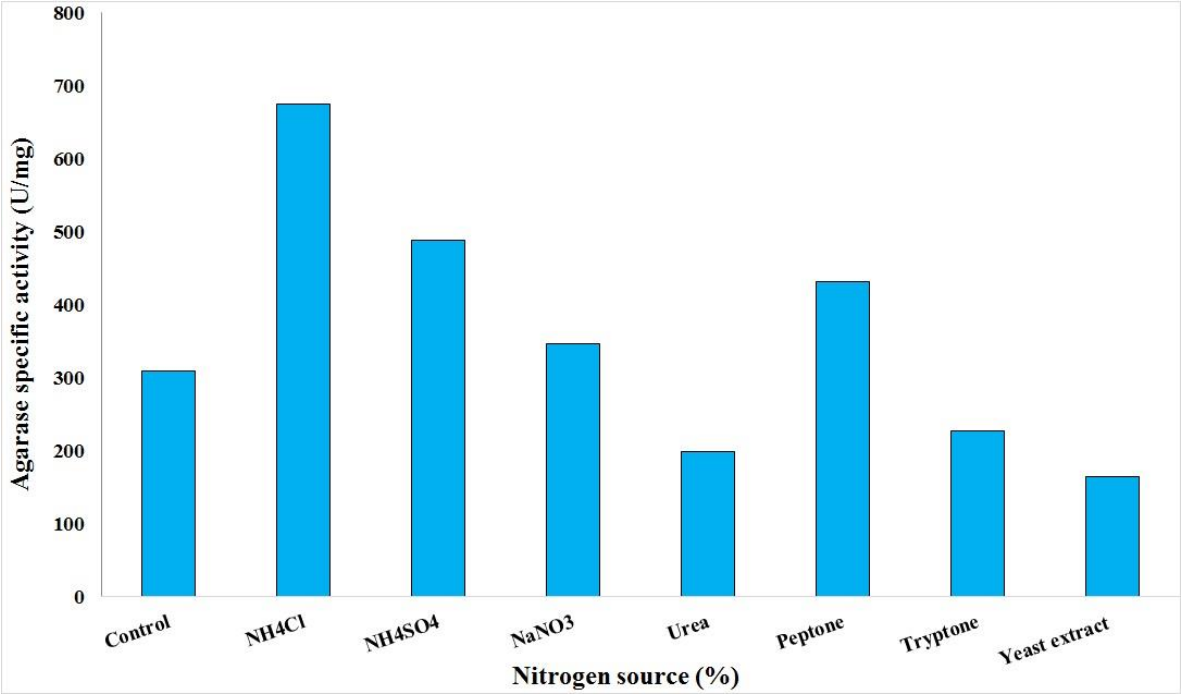

Fig. 3: Effect of nitrogen sources on agarase production.

3.2. Effect of Culture Condition on Agarase Production: The effect of culture conditions on agarase production of *Bacillus* sp. R2 in shake flasks (submerged fermentation), using one factor at time technique (OFAT) were summarized in Table (1). A 75% (v/v) sea water strength and 5% NaCl were the most appropriate dilution and concentration, respectively for the optimum agarase production. Furthermore, the initial pH: 7, temperature 30 °C, inoculum size 5%, and 150 rpm/min shaking rate also gave the highest agarase activity. From the available scientific literatures, it seemed that agarase fermentation conditions were microbe to microbe variable. For example, the optimal temperature and inoculum size were reported to range from 20 to 37 °C, and from 1 to 5%, respectively [22, 24, 25]. Furthermore, Fu *et al.* [21] obtained the maximum agarase activity from *Agarivorans albus* YKW-34 in the following fermentation condition, 7.8 initial pH, 25 °C temperature, $1* 10^7$ CFU/ml inoculum size, and 120 rpm/min shaking rate.

Table (1): Effect of Fermentation conditions on agarase production.

Parameter	Value	Agarase specific activity(U/mg)
Sea water strength (%)	25	162.23
	50	254.26
	75	490.37
	100	315.32
Salinity (NaCl %)	2.5	308.28
	5	344.57
	7.5	290.78
	10	180.97
Inoculum size (%)	2.5	452.1
	5	534.28
	7.5	272.47

	10	126.35
pH	5	240.25
	6	310.98
	7	522.48
	8	481.24
	9	290.87
	10	121.29
Temperature (C°)	20	310.58
	30	490.54
	37	419.35
Agitation rate (rpm)	0	130.54
	50	205.66
	100	362.24
	150	522.49
	200	396.57

4. Conclusions

The optimization of medium constitution specially carbon and nitrogen sources (0.2% agar and 0.2% lactose as essential and secondary carbon sources, 0.5% NH4Cl) as well as fermentation conditions (5% NaCl, 5% culture seed, pH: 7, 30 °C, and 24 h incubation period at 150 rpm shaking.) significantly enhanced agarase production by more than 2 folds, this cost-effective optimization help understanding *Bacillus* sp. R2 agarase production physiology, and recognizing the key influencing factors, which may pave the way for the industrial scaling up with low cost and high enzyme yield.

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Conflicts of Interest: The author declares that there is no conflict of interest.

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