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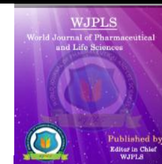

VIBRIO SP. R1 AGARASE: SCREENING, PRODUCTION AND SUBSTRATE SPECIFICITY

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ABSTRACT

Forty-two bacterial strains isolated from marine and terrestrial sources and were tested for agarase productivity. After successive rounds of primary and secondary screening, an agar-degrading marine bacterium from the Red Sea (Hurghada- Egypt), designated R1 attracted our attention by its strong agarovorant and high chitinolytic activity. The morphological, physiological and biochemical characterization showed that the strain was gram negative, facultative anaerobe, motile curved rod, oxidase, catalase urease and O–F test positive. Furthermore, produced acid but no gas from glucose, therefore, it was assigned to the genus *Vibrio*. Time course of growth, agarase and chitinase production and co-production by Vibrio sp. R1 were determined, Furthermore, the substrate specificity of crude extracellular agarase against different polysaccharides was evaluated. *Vibrio sp*. R1 crude agarase preparation showed height activity for degrading certain complex polysaccharides other than agar and agarose including shrimp shell colloidal chitine, crab shell chitosan, cellulose, carboxy methyl cellulose, pectin, alginic acid and Arabic Gum. Due to the large number of the hydrolytic enzymes produced by *Vibrio sp*. R1 and particularly the multi polysaccharides degrading activity of their agarases. We suggest that bacteria play an important ecological role in organic material recycling and mineralization at the edges of the seas and oceans. In addition, the agarase hyperactivity enlighten the potential application of *Vibrio sp*. R1 as promising new source for possible biotechnological applications.

KEYWORDS: *Vibrio sp*. R1, isolation, identification, agarase, production, substrate specificity.

INTRODUCTION

Agar is a complex polysaccharide derived from the agarophytes of the Rhodophytea such as *Gelidium sp, Gracilaria sp and Petrocladia sp*., consisted of agarose and agaropectin. Agarose is linear polysaccharide composed of alternating residues of 3-0-linked ß-D-galactopyranose and 4-0-linked 3.6–anhydro–α– l –galactopyranose, while agaropectin is a polysaccharide containing substituent groups such as sulfate, methoxy and pyruvate.[1]

During the last decades agarases attracted the attention of many researchers due to their extended versatile biotechnological applications ranging from of algal biomass valorization for fuel, feed and food to cosmeceutical, pharmaceutical and medical biotechnology.[2,3,4,5,6]

Agarases are glycoside hydrolases (GH) consisting of α-agarases (EC 3.2.1.158) and β-agarases (EC 3.2.1.81), they have been isolated from mollusks (2) and diverse bacterial genera found in seawater, marine sediments and other environments.[7,8,9,10,11,12,13] furthermore Vibrio agarases production, purification and characterization were wealthy reported,[14,15,16,17,18] whereas agarase substrate specificity have not been fully investigated, for this reason, the present study was undertaken with the aim of isolate, screen, and identify marine agarovorant chitinolytic bacterium *Vibrio sp.* R1, furthermore co-produce and investigate the effect of their crude agarases on different polysaccharides.

MATERIAL AND METHODS

Chemicals

Galactose, agarose, crab shells chitosan, bovine serum albumin (BSA), Sodium alginate, starch (Sigma -USA), crystalline cellulose, CMC (BDH), agar, Bactopeptone, and yeast extract (Oxoid Hampshire, England). blue dextran 2000 (Pharmacia-Sweden), Gum Arabic (Janssen Chemica- Belgium), gelatin and lactose (Adwec -Egypt). apple pectin (Fison- Germany), crab shells chitine (win lab -UK), 2 Hydroxy 3,5 dinitrosalselic acid (DNSA) (Merck, Darmstadt- Germany), All other reagents were of analytical grade and commercially available.

Sampling and isolation of agarolytic bacteria
Water, sands and sediments samples from Mediterranean and red seas and Mariout lake, Nile River (Egypt) were collected, and 0.1 ml were spread directly, whereas soil, sewage, and wastewaters samples were diluted in sterilized sea water and plated on agarase detection medium (ADM) composed of 1.5% Agar as sole carbon source, 0.05% yeast extract dissolved in sea water (pH 7.4) and incubated at 30°C for few days (48 -120 h).

Primary Screening for agarolytic bacteria
Performed on (ADM) solid medium, after 5 days, agarolytic activity were assessed firstly by liquefaction or shallow depression appearing around the colonies, and secondly, via plate flooding with Lughole's solution (0.1 %. I_2 and 0.6 % KI) and the appearance of pale-yellow zones around colonies against a reddish-brawn background was considered indicative for agarolytic activity. All colonies that formed depression or pits on the agar plate surface, or maximum zone of clearance, were purified by streaking on the same medium and picked up for further analysis.

Secondary Screening for agarolytic bacteria
The isolate showing the higher ratio of clearing zone to colony size from the primary screening were chosen for secondary screening via testing their productivity on agarase production liquid medium (0.2% Agar, 0.3% yeast extract dissolved in filtered sea water. pH: 7.2) and incubated for 24 h at 30 ^{o}C and 180 rpm shaking rate.

Identification of agarolytic potent strains
Screening resulted potent isolate were identified according to the morphological, physiological and biochemical tests described in Bergey's manual of systematic Bacteriology.[19]

Agarase and chitinase production and co-production
A single colony was inoculated 5 ml of marine LB and grown at 30 C^{o} on rotary shaker for 24 h. The resulting overnight culture was then transferred into 250 ml Erlenmeyer flask containing separately 50 ml of agarase and chitinase production media prepared with sea water (0.2% Agar, 0.3% yeast extract, pH: 7.2) and (0.5% colloidal chitin, 0.2 % yeast, pH: 7.5), respectively, the flasks incubated at 30 ^{0}C with 180 rpm agitation rate, the changes in the enzyme activity, protein content and growth were monitored each 12h for 3 days. The co-production was conducted in sea water supplemented with (0.2% Agar, 0.2% colloidal chitin and 0.3% yeast extract, pH: 7.2).

Crude agarase substrate specificity
After 24 h of incubation, the co-production medium was centrifuged at 8000 rpm for 15 min at 4 °C and the supernatant collected was taken as a crude extracellular enzyme. The substrate specificity of the crude agarase against the different polysaccharides was evaluated under the same condition and buffer sited below instead agar the desired polysaccharide was tested (agarose, shrimp shell colloidal chitine, crab shell chitosan, cellulose, carboxy methyl cellulose, starch, pectin, Arabic Gum, alginic acid). Relative activity (RA) was calculated according to the following formula[20] using the standard assay as the control.

$$\text{Relative activity (RA)} = \frac{\text{EA in control} - \text{EA in treated}}{\text{EA in control}} \times 100$$

Where, EA is the enzyme activity.

Analytical methods
1. Agarase assay
Agarase activity was analyzed according to the method of (Miller, 1959),[21] by estimating the reducing end sugars, the assay was performed by mixing 0.5 ml of cell free supernatant (crud Enzyme) with 0.5 ml bacto agar solution (0.1% Bacto gar, 50 mM tris - Hcl, pH 7.2, 5 mM cacl2, 100 mM Nacl, at 37 oC) at the end of incubation (30 min). hydrolysis reactions were terminated and analyzed by adding 1ml of dinitro salicylic acid reagent, the mixture was boiled for 5 min, chilled and diluted with 10 ml distilled water, the resulting adduct of reducing sugars were measured spectrophotometrically at 540 nm. One unit (U) of the enzyme was defined to be the amount that catalyzed the liberation of 1 μmol of reducing sugar per minute under the above conditions using galactose as standard. The specific activity was expressed in one mmol galactose equivalents/min/mg protein.

2. Chitinase assay
Chitinase activity was analyzed by estimating the released reducing ends of sugar according to the method of (Miller, 1959),[21] using N-acetyl - D-glucosamine (NAG) as standard. One unit of chitinase activity was defined as the amount of enzyme required to release 1 μ mol of NAG per minute during these conditions.

3. Growth monitoring and Protein assay
The bacterial cell growth was monitored spectrophotometrically by measuring the absorbance of the cultures at 660 nm. as described in (Bradford,1976) method.[22] Soluble proteins were quantified using bovine serum albumin as a reference for preparing the standard curve.

RESULTS AND DISCUSSION

Agarolytic potent strain Screening, isolation and identification
The 42 agarolytic strain isolated from different sources were subjected for primary screening which reveled on 11 potent strains showing the higher ratio of clearing zone to colony size, this later were chosen for secondary

screening. The results illustrated in (Fig.1) showed eight isolates produced agarase and chitinase within 24 h, the marine agarolytic isolate R1 was the most potent strain chowing strong agarovorant and chitinolytic activity (Fig.2) consequently it was selected for further analysis and identification.

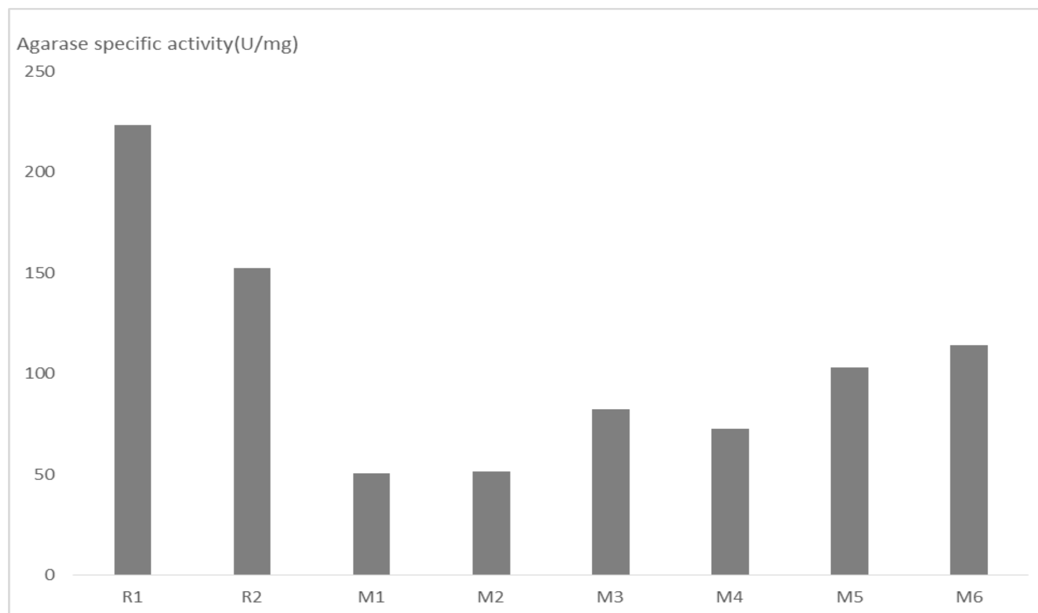

Fig. 1: Secondary screening of agarase producing bacterial isolates.

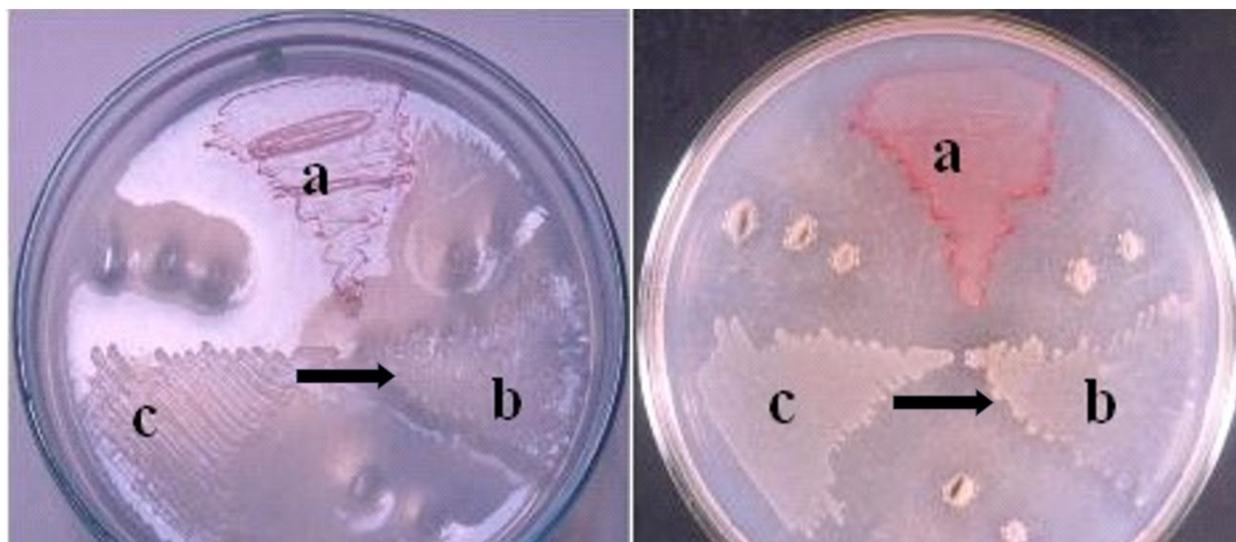

Fig. 2: the agarovorant (left arrow) and chitinolytic (right arrow) activities of Vibrio sp. R1 isolate(b), a and c were other chitinolytic strains.

Agarolytic potent strains identification

Among the 42 screened bacterial isolates, the red sea isolates R1 (Hurghada- Egypt) was marine bacterium exhibiting agarovorant and high chitinolytic activity enable it as excellent candidate for agarase and chitinase co-production. The morphological, cultural, as well as physiological and biochemical characteristics summarized in Table (1): showed that the strain was gram negative, facultative anaerobe, motile curved rod does not form endospores, oxidase, catalase urease and O–F test positive, the growth occurs optimally at 30°C, pH 7 and 3% NaCl with a range of 4-40°C, pH 5-10, and NaCl 2-10 %, respectively. Furthermore, produced acid but no gas from glucose, produced several enzymes such as alginase, lipase, amylase, protease, gelatinase, and β-galactosidase, therefore, it was assigned to the genus Vibrio.

Many investigators reported agarase production by *Vibrio*.[14,15,16,17,18]

Table (1): Morphological, physiological and biochemical characteristics of Vibrio sp. R1 isolate.

Characteristic	Result	Characteristic	Result	Characteristic	Result
Gram staining	-	Agar	+	65°	-
Form or shape	Cu. rod	Chitin	+	Growth in NaCl	
Spore	-	Starch	+	2 %	+
Pigment production	-	Crystalline cellulose	-	5%	+
Motility	+	CMC	+	7%	+
Aerobe/anaerobe	+/+	Pectin	-	10%	+
O/F test	+/+	Alginate	+	acids from	
Oxidase	+	Arabic gum	+	Glucose	+
Catalase	+	Dextran	-	Galactose	Alk.
Urease	+	Urea	+	Fructose	-
Nitrate reductase	+	Tween 20 & 80	+	Mannose	+
Methyl red (MR)	-	Casein	+	Arabinose	-
Voges-Proskauer (VP)	-	Gelatin	+	Xylose	-
Indole	-	Growth at:		Sorbose	-
Citrate	-	4°	+	Lactose	-
Growth in Mac koncy	+	20°, 37°-40°	+	Sucrose	+
Hemolysin	-	55°	-	Glycerol	+

Cu: curved, w: weak, vw: very weak. Alk: alkaline reaction

Agarase and chitinase production

Time course of growth, agarase and chitinase production by Vibrio sp. R1 in the preoptimized media were determined (Fig. 3 and 4). The amount of agarase and chitinase production increased synchronously and rapidly from 12 h to achieve their maximum at 24 h then decreased gradually over the 72h. agarase production from vibrio within 1 day were reported in many literatures.[14,15,16,17,18] However, no works related to chitinase and agarase co-production by vibrio were reported. The Fig.3 and 4 showed that the agarase secreted during the exponential phase but the maximal production of the enzyme was obtained it late log phase in contrast (Vera *et al*. 1998),[23] found that the highest level of agarase from *Pseudoalteromonas* was reached during the stationary phase, and at longer incubation periods the level of agarase and chitinase decreased, a trend probably due to the presence of proteases processing regulation.

Both glucose, galactose and lactose were tested as inducers for agarase production, and they were effective carbon sources for enzyme production (data not shown) in contrast (Day and Yaphe, 1975),[24] found that glucose repress the agarase production, but galactose induce them, in addition (Agbo and Moss, 1979),[25] reported that both glucose and galactose repressed agarase from *Cytophaga* sp and *Alteromonas* sp.

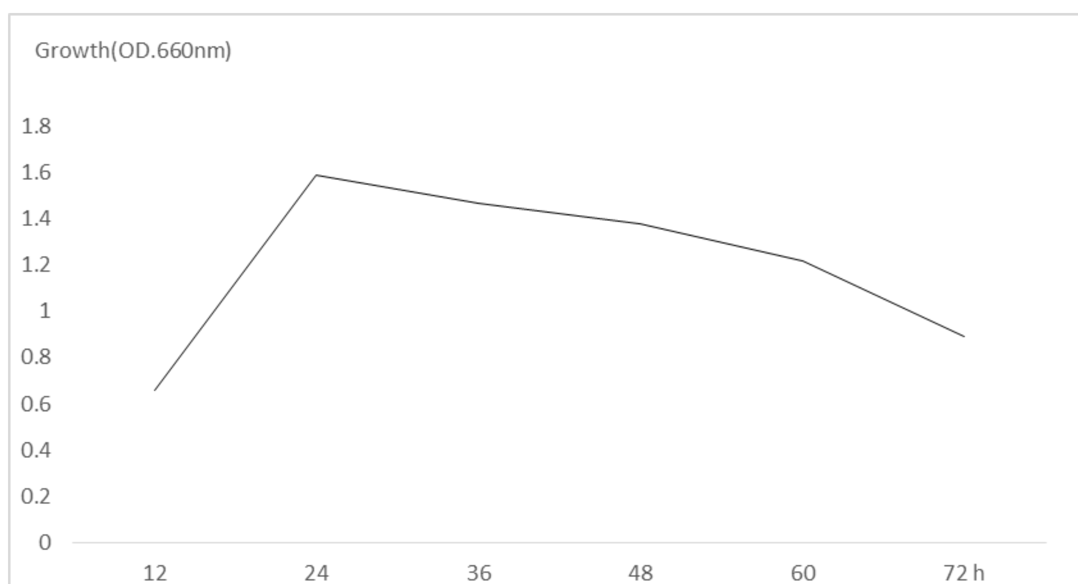

Fig. 3: *Vibrio sp*. R1 growth curve.

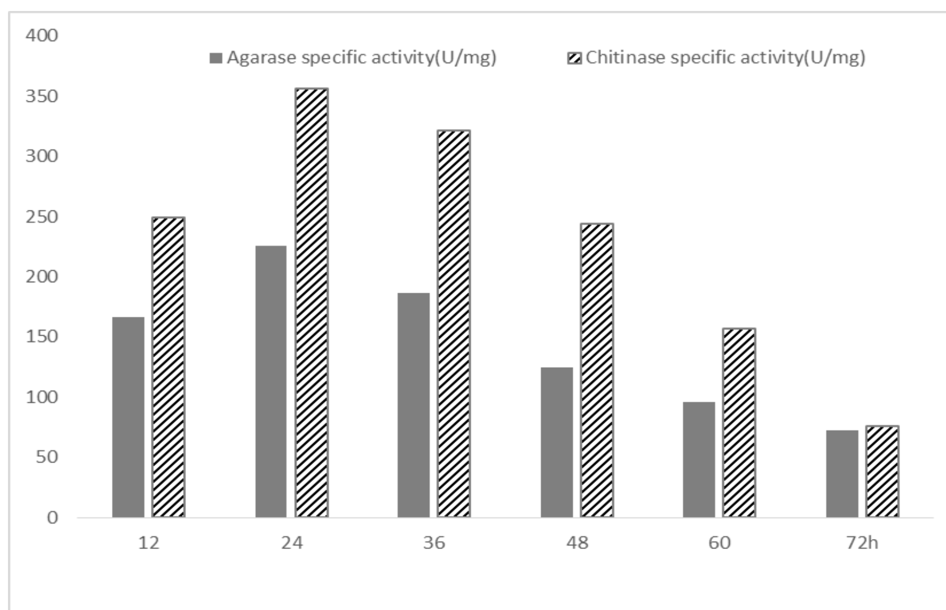

Fig. 4: Time course of agarase ad chitinase production by *Vibrio sp*. R1.

Crude agarase substrate specificity

After 24 h of incubation, the agarase and chitinase co-production medium was centrifuged at 8000 rpm for 15 min at 4 °C and the collected supernatant was subjected to well-cut diffusion assay on agarose plates assessed using Lughole's solution to confirm the agarase activity (Fig.5) the clear halo formed around the well indicated the strong diffusion of agarase and releasing reducing compounds. Furthermore, the substrate specificity of this crude extracellular agarase against the different polysaccharides was evaluated (Fig.6) Vibrio sp. R1 crude agarase preparation showed height activity for degrading certain complex polysaccharides other than agar and agarose including shrimp shell colloidal chitine, crab shell chitosan, cellulose, carboxy methyl cellulose, pectin, alginic acid and Arabic Gum, the crud agarase did not degrade crab shall chitin due it's very hard and crystalline structure, and show low activity in degrading starch in addition to ß-galactosidase activity on lactose. The moderate degradation of colloidal chitin and chitosan may be due the chitinolytic enzymes induced in the co-production medium.

Similar activities were obtained by the agarase purified by(Stoz *et al.* 2001).[26] from marine bacterium (*Alteromonas* stain 2–40) which depolymerize wide range of polysaccharides such as alginate, CMC, chitine, glycogen, laminarin, pullulan, starch, xylan, sodium polygalacturonate, in particular agar and agarose however The 2–40 agarase enzyme system selectively does not digest carrageenan, cellulose, dextran, inulin, pectin and poly galacturonic acid, in contrast (Vander meulen and Harder, 1975).[27] found that the agarase of *Cytophaga flevensis* was very specific, hydrolyzing agar, agarose and agaropectin but not porphyran, carrageenan, pectin, poly galactoaraban, inulin or amylose, also (kiremura *et al.* 1999),[9] found that the agarase from *Alteromonas* sp. E-1 did not degrade sodium alginate nor κ- carrageenan and it was very specific to agar and agarose like the agarase of *Agartacterium pastinator*.[28] in other hand (Liu *et al.* 2016).[29] reported that a multifunctional amylase Amy63 from *Vibrio alginolyticus* possessed amylase, agarase, and carrageenase activities.

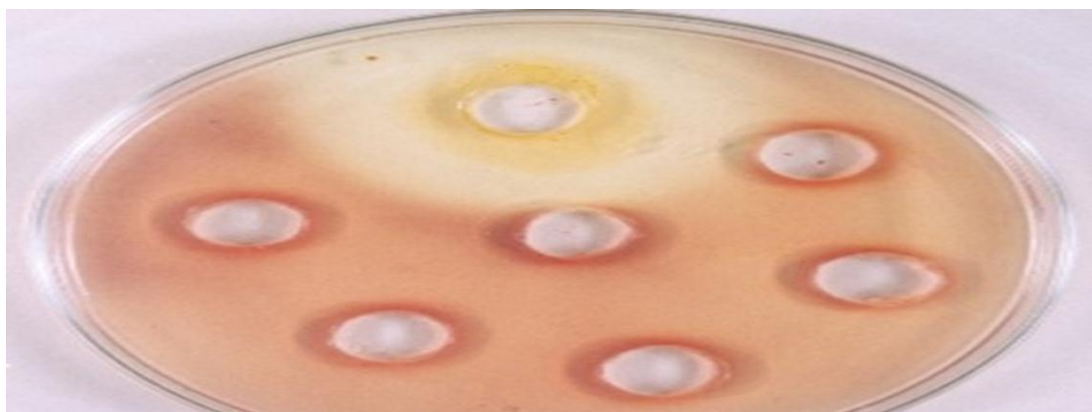

Fig. 5: *Vibrio sp*. R1 agarase activity, through well cut diffusion assay on agarose plates assessed using Lughole's solution, the positive result confirmed by clear zone around well against a light reddish-brawn background.

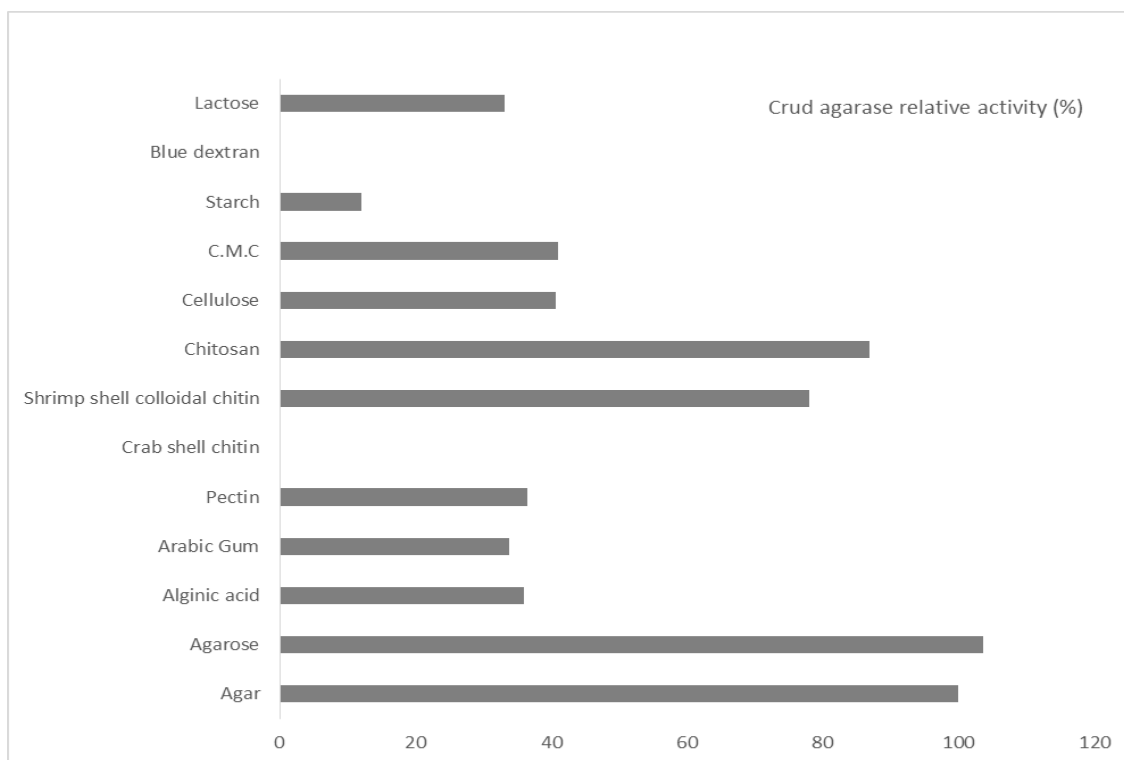

Fig. 6: *Vibrio sp*. R1Agarase crude agarase substrate specificity.

CONCLUSION

Due to the large number of the hydrolytic enzymes produced by *Vibrio sp*. R1 and particularly the multi polysaccharides degrading activity of their agarases, therefore, maybe play an important ecological role in organic material recycling and mineralization at the edges of the seas and oceans. Furthermore, this strain can be a good candidate for agarase production and may pave the way for algal biomass valorization for versatile biotechnological applications.

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