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Isolation and Optimization of Agarase Enzyme by Marine Bacterium Bacillus Subtilis

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Abstract

Agarolytic bacteria were isolated from the coast of the Mediterranean Sea of New Damietta City, Damietta Governorate, Egypt by enrichment culture technique on nutrient agar medium dissolved in seawater. Totally 32 morphological different bacterial colonies were isolated. On Basal salt solution medium B, all of the isolates were tested for agarase activity. Lugol's iodine solution was used to detect agarase activity. Among the 32 isolates, 12 isolates showed agarase activity. Agarase activity was measured using the DNS method. Maximum agarolytic activity was recorded in the isolate D8. The identity of the agarolytic isolate (D8) was confirmed based on the microscopical, morphological, and biochemical tests. The bacterium was identified as *Bacillus subtilis*. The optimization of culture conditions was carried out for the ideal *Bacillus subtilis* growth and extracellular agarase production. The fermentation time, temperature, pH, and NaCl concentration that *Bacillus subtilis* needs to produce maximum agarase amount were 48 hrs, 40℃, 8, and 3 % concentration respectively. The optimum carbon source for agarase production was found to be Agar (0.4%) among the examined carbohydrates. The best nitrogen source for *Bacillus subtilis* agarase production was yeast extract (0.3%).

Keywords: Isolation, Optimization, Agarase, Marine, Bacillus subtilis.

Introduction

The marine environment not only contains a huge diversity of creatures, but it also provides a prospective supply of microbes with great potential (Beygmoradi & Homaei, 2017). When compared to their terrestrial homologs, the distinctive characteristics of marine microorganisms can be linked to their various genetic architecture, physiological adaptations, and metabolic variations (Barzkar et al., 2018). Novel enzymes with interesting biotechnological uses can be found in abundance in marine microorganisms. Therefore, compared to their terrestrial counterparts, marine microbial enzymes have more benefits, such as more biochemical variation, genetic modification, the capacity for mass culture, increased catalytic activity, and sustainability (Bull et al., 2000). Agarases are

one of the most important enzymes generated by marine microorganisms. Agarases are hydrolytic enzymes that are capable of catalyzing the degradation of structured polysaccharides such as agar and creating bioactive oligosaccharides with a variety of functional activities and potential industrial uses (Chen et al., 2005; Delattre et al., 2011; Kim et al., 2012; Wang et al., 2020). Based on how the bacteria affect solid agar, agarolytic bacteria are divided into two classes. The bacteria in class one softened agar, creating depression all around it, while class two includes bacteria that lead to the liquefication of agar (Sharabash et al., 2022). Agar is the primary building block of red algae Rhodopyhta cell walls (Rajeswari et al., 2016). An et al. (2018) and Han et al. (2016) discovered that a great number of agar-degrading bacteria are naturally prevalent in marine environments. Numerous microbes isolated from marine environments have been found to exhibit agarase activity; they include *Alteromonas* (Chi et al., 2014a), *Pseudoalteromonas* sp. (Chi et al., 2014b), *Micrococcus* (Choi et al., 2011), *Thalassomonas* (Ohta et al., 2005), *Vibrio* (Liu et al., 2020), *Microbulbifer* (Jonnadula & Ghadi, 2011) and *Agarivorans* (Hu et al., 2009). A small number of isolated bacteria have been described from non-marine habitats, including *Bacillus* sp. (Suzuki et al., 2003), *Streptomyces coelicolor* A3(2) (Temuujin et al., 2012), and *Streptomyces lavendulae* UN-8 (Wu et al., 2017) that isolated from the soil. *Asticcacaulis* sp.SA7 from rhizosphere soil (Hosoda & Sakai, 2006). Agarose and agaropectin are the two polysaccharides that form agar (Kolhatkar & Sambrani, 2018; Kwon et al., 2020). The linear polysaccharide known as agarose is made up of β-D galactose and 3,6-anhydro-α-L-galactose units connected by α-1,3 and β-1,4 glycosidic bonds (Fu & Kim, 2010). Similar to agarose, agaropectin consists of basic disaccharide-repeating units; however, methoxy, pyruvate, glucuronate, or sulfoxy groups partially substitute the hydroxyl groups of the 3,6-anhydro-α-L-galactose units (Lee et al., 2023; Mandal et al., 2023). Agarases are classified into two categories based on their cleavage pattern: β-agarases (EC 3.2.1.81) and α-agarases (EC 3.2.1.158) (Li et al., 2020). β-agarases break down the β-1,4 glycosidic linkages in agar and form neoagarooligosaccharides that have D-galactose residues at the ends that reduce, whereas α-agarases break down the α-1,3 glycosidic linkages in agar and form agarooligosaccharides that have 3,6-anhydro-L-galactose residues at the ends that reduce (Park et al., 2020). The agarase enzymes may exhibit endo- or exo-activities, or both (Chi et al., 2012; Roseline & Sachindra, 2018). The majority of agarases are produced extracellularly, and the other is a few agarases produced intracellularly (Fu & Kim, 2010). β-agarases exhibit a higher prevalence compared to α-agarases (Jahromi & Barzkar, 2018). Agar hydrolysis is just one of the many applications for agarase enzymes to produce oligosaccharides with physiological properties that are useful in food, cosmetic, and pharmaceutical applications, such as anti-obesity (Hong et al., 2017), anti-inflammatory (Wang et al., 2017), agents for skin whitening with nourishing qualities (Kim et al., 2017), antioxidant (Zhang et al., 2019), prebiotic (Han et al., 2019; Xie & Cheong, 2022). Agarases are also used to make marine algal protoplasts (Araki et al., 1998). Also, they are used to extract agarose gel DNA (Zhang & Sun, 2007). The requirement to dispose of a lot of agar medium after use makes agarases essential in research labs as well. The agar solidifies, clogging the drainage systems. Therefore, it's critical to preserve agarolytic bacteria that are employed to treat this issue and sustainably dispose of agar (Kolhatkar & Sambrani, 2018). Nutritional sources and growth conditions have a significant impact on the microbe's ability to produce enzymes (Philip et al., 2020). To achieve a high yield of product and reduce the cost of the fermentation process, an efficient production medium needs to be constructed (Veerakumar & Manian, 2022). This can be done by optimizing the production medium. Among the techniques used to determine the ideal parameters and their precise impacts is the One Factor At-Time (OFAT) technique (Shah & Mishra, 2020). In OFAT, one parameter is changed while keeping all other parameters constant to determine how that parameter affects the response. Increasing the activity of the produced enzyme depends critically on optimizing every factor in the stage of fermentation. The present investigation aimed to isolate and optimize an agar hydrolytic marine bacterium *Bacillus subtilis*.

Material and methods:

Water samples collection:

The samples of water were gathered from the Mediterranean Sea coast of New Damietta City, Egypt. In sterilized glass bottles water samples were collected from various locations and depths. All samples of water were collected and sent to the Microbiology Lab at Damietta University's Faculty of Science for marine bacteria isolation.

Water samples enrichment:

To promote the development of agarolytic bacteria, the samples were enriched using the enrichment culture method. Each water sample received 100 ml of agar 0.2% (w/v) as the only source of carbon and was autoclaved (121°C, 1.5 atm, 20 min). Then, under aseptic circumstances, 10 ml of saltwater (inoculum) was introduced to each sample. Following that, all samples were stored in an aerobic shaker incubator (4 days, 30°C, 150 rpm) (Agbo & Moss, 1979).

Isolation of marine water bacteria:

Serial dilutions of the enriched samples (10^{-1} to 10^{-5}) were performed, and 0.1 ml aliquots of each dilution were subsequently cultivated on nutrient agar media containing (g/L): peptone (5g), yeast extract (3g), and Agar (20g) which dissolved in 1 L of seawater (pH was 7.5). The plates were kept at 30°C for 48 hours of incubation. After the incubation period, the bacteria were further cultivated using the quadrant streak method on the same medium to purify the single colonies. All the isolated purified bacterial strains were kept on a slant culture at 4°C for further use (Sharabash et al., 2022).

Screening of all isolates for the production of agarase enzyme:

Screening of agarolytic activity of all isolated bacteria was performed on basal salt solution medium B (Hofsten & Malmqvist, 1975) containing (g/L dist.H_2O): $NaNO_3$ (2g), K_2HPO_4 (0.5g), $FeSO_4.7H_2O$ (0.02g), $MgSO_4.7H_2O$ (0.2g), $CaCl_2.2H_2O$ (0.02g), $MnSO_4.7H_2O$ (0.02g), and Agar (20g) which was adjusted to pH 7.5 before autoclaving. After the autoclaving process, all the isolated bacteria were inoculated by the streak plate method on the screening medium and incubated at 30°C for 2 days. Lugol's iodine solution was employed to identify agarase enzyme qualitatively, hence all growth plates were flooded with iodine solution (1g I_2, 2g KI, 100 ml dist H_2O.). The presence of a clear zone surrounding the colony shows that it may degrade agar. The microorganisms with agarolytic activity were chosen for further research.

Determination of agarase activity:

The 3,5-dinitrosalycilic acid (DNS) method was used to determine agarase activity quantitively (Miller, 1959). This method was used to measure reducing sugar amounts released from agar hydrolysis. All the selected isolates of agarolytic activity were cultured in 100 ml Erlenmeyer flasks containing 50 ml of screening medium that provided with agar 0.1% (w/v) as the only source of carbon and then incubated for 48 hrs at 30°C in a shaking incubator (150 rpm). Cultures were then centrifuged (4000 rpm, at 4°C for 15 min) to discard the cells of bacteria and obtain supernatant which was used as the extracellular enzyme source.

Assay of agarase enzyme:

A 3 ml solution of Tris HCl buffer (20 mM, pH 8) containing 0.2% (w/v) agarose as substrate was mixed with 1 ml of supernatant. The incubation of the mixture reaction occurred for 30 min at 40°C. After taking 1 ml of the reaction and adding 1 ml of DNS to it, the reaction was heated for 10 mins to boiling water to stop it. After cooling at room temperature, the reducing sugar product's absorbances were determined spectrophotometrically (λ=540nm). Blanks of enzyme with no substrate and substrate with no enzyme were treated in the same way. The standard curve was developed using (D-galactose) as the standard reducing sugar in the range (0–10µmole/ml). The enzyme quantity needed to release 1µmole of reducing sugar (D-galactose) per mint at the conditions of the assay is known as One unit of enzyme activity (U) (Saravanan et al., 2015).

The isolated marine bacteria identification:

Gram and endospore staining were applied to stain the isolated purified bacteria. The identification was based on the characteristics

of the colony; size, shape, elevation, margin, surface, color, and opacity. The biochemical tests were assessed according to Bergey's Manual of Systematic Bacteriology (Sneath, 1986) for the potential producer isolate of agarase enzyme.

Optimizing culture conditions to promote growth and production of agarase enzyme:

The higher producer isolate for agarase enzyme was selected for determining the effect of physicochemical parameters to maximize the production of agarase and bacterial growth. The major parameters in this investigation were temperature, pH, incubation time, NaCl concentration, and various sources of carbon and nitrogen. Every parameter was adjusted through the fermentation process and fixed in the stages that followed to optimize the others. Each optimization experiment was carried out in triplicates (Jung et al., 2012; Ziayoddin et al., 2014).

The incubation period impact on bacterial growth and agarase production:

The incubation period was studied for 5 days with a 1-day interval on the screening medium containing 0.2% (w/v) agar. To evaluate the bacterial growth, The fluid culture's optical density was measured spectrophotometrically (λ=600nm), and the assay of agarase enzyme was determined as described before.

The temperature impact on bacterial growth and agarase production:

The screening medium containing 0.2% (w/v) agar was incubated at various temperatures (25-55°C) to study the effect of temperature. Bacterial growth and agarase activity were measured as previously described.

The pH impact on bacterial growth and agarase production:

To investigate the impact of pH, the screening medium containing 0.2% (w/v) agar was adjusted by 1N HCl or 1N NaOH to different pH ranges (3-12) with one pH unit interval. The bacterial growth and agarase activity were recorded as described before.

The NaCl concentration impact on bacterial growth and agarase production:

The concentration of NaCl was investigated within a range of 0 - 5% (w/v) at intervals of 0.5% on a screening medium that contained 0.2% (w/v) agar. Agarase activity and bacterial growth were recorded as described before.

The various carbon sources impact on bacterial growth and agarase production:

To investigate the impact of various carbon sources, a range of sugars such as (agarose, galactose, glucose, fructose, rhamnose, sucrose, lactose, maltose, trehalose, mannitol, raffinose, arabinose, xylose, cellulose, and xylan) were added in amounts equal to 0.2% (w/v) of agar. The bacterial growth and agarase activity were recorded as described before. The optimum carbon source concentration was studied by the addition of various concentrations in a range of 0.1-1% (w/v) with 0.1% intervals separately on the screening medium (Saraswathi et al., 2011).

The various nitrogen sources impact on bacterial growth and agarase production:

A variety of nitrogen sources such as (NH_4NO_3, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, $NH_4H_2PO_4$, NH_4Cl, yeast extract, peptone, beef extract, tryptophane, tryptone, glycine, and casein) were added in the quantities required to provide the equimolecular amounts of nitrogen found in $NaNO_3$ (0.2%) used in the basal salt solution medium B. The bacterial growth and agarase activity were recorded as described before. The optimum nitrogen source concentration was studied by the addition of various concentrations in a range of 0.1-1% (w/v) with 0.1% interval separately on the screening medium (Saraswathi et al., 2011; Ziayoddin et al., 2014).

Statistical analysis:

The statistical analysis was carried out using the SPSS software version 18. The data was examined using one-way analysis of variance (ANOVA). To establish statistical significance, a *p*-value of less than 0.05 was employed. Every experiment was done in triplicate. The data was given as mean± standard error.

Results:

Isolation of marine water bacteria and screening of agarolytic activity:

In total, 32 bacterial isolates were isolated using a nutrient agar medium that dissolved in seawater. These isolates were identified according to their microscopic and morphological characteristics. All the isolates were screened for the production of agarase enzyme. It was found from all the isolated bacteria, only 12 isolates could produce agarase enzyme **(Table 1)**. The twelve isolates were assayed for measuring the agarase activity. Isolate D8 was found to be the potential producer of agarase and screened for its agarolytic activity as shown in **Figure 1**.

Table 1. Morphological characteristics of isolated marine bacteria and screening of agarolytic activity.

Isolate number	Colony characteristics						Cell characteristics				Agarase production
	Size	Shape	Margin	Elevation	Color	Surface	Opacity	Gram stain	Shape of cell	Endospore stain	
D1	Medium	Round	Entire	Flat	White	Smooth	Opaque	Positive	Cocci	Negative	Negative
D2	Large	Round	Entire	Flat	Creamy	Smooth	Opaque	Positive	Cocci	Negative	Negative
D3	Large	Round	Irregular	Flat	White	Smooth	Opaque	Negative	Bacilli	Negative	Positive
D4	Small	Round	Entire	Flat	Off white	Smooth	Opaque	Positive	Cocci	Negative	Negative
D5	Medium	Round	Entire	Flat	Off white	Smooth	Opaque	Positive	Bacilli	Negative	Negative
D6	Small	Punctiform	Entire	Flat	white	Smooth	Transparent	Positive	Bacilli	Negative	Positive
D7	Small	Round	Entire	Flat	Yellow	Smooth	Opaque	Positive	Cocci	Negative	Negative
D8	Medium	Irregular	undulate	Flat	creamy	Rough	Opaque	Positive	Bacilli	Positive	Positive
D9	Medium	Round	Entire	Convex	White	Mucoid	Opaque	Positive	Rods	Negative	Positive
D10	Medium	Round	Entire	Convex	Orange	Smooth	Opaque	Positive	Cocci	Negative	Negative
D11	Small	Round	Entire	Convex	Beige	Smooth	Opaque	Negative	Rods	Negative	Negative
D12	Small	Round	Entire	Flat	White	Mucoid	Opaque	Positive	Bacilli	Negative	Negative
D13	Small	Round	Entire	Flat	Yellow	Rough	Translucent	Negative	Rods	Negative	Negative
D14	Small	Round	Irregular	Convex	Off white	Mucoid	Opaque	Negative	Rods	Negative	Positive
D15	Small	Round	Entire	Flat	White	Smooth	Opaque	Negative	Bacilli	Negative	Positive
D16	Small	Round	Entire	Convex	Yellow	Smooth	Opaque	Positive	Cocci	Negative	Negative
D17	Small	Round	Entire	Flat	White	Rough	Opaque	Positive	Bacilli	Negative	Negative
D18	Small	Round	Entire	Convex	White	Smooth	Opaque	Positive	Bacilli	Negative	Negative
D19	Medium	Round	Entire	Convex	Milky	Smooth	Opaque	Positive	Bacilli	Positive	Positive
D20	Small	Round	Entire	Flat	Creamy	Rough	Opaque	Positive	Cocci	Negative	Negative
D21	Medium	Round	Entire	Flat	White	Rough	Opaque	Positive	Rods	Negative	Positive
D22	Small	Rhizoid	Lobate	Flat	Creamy	Rough	Opaque	Positive	Rods	Negative	Negative
D23	Small	Punctiform	Entire	Flat	White	Smooth	Opaque	Positive	Cocci	Negative	Negative
D24	Small	Round	Entire	Flat	Red	Smooth	Opaque	Negative	Rods	Negative	Positive
D25	Large	Round	Entire	Flat	White	Rough	Opaque	Positive	Bacilli	Negative	Negative
D26	Small	Round	Entire	Flat	Pale yellow	Smooth	Opaque	Positive	Rods	Negative	Negative
D27	Small	Punctiform	Entire	Flat	White	Smooth	Transparent	Positive	Cocci	Negative	Negative
D28	Small	Round	Entire	Convex	Beige	Smooth	Opaque	Positive	Rods	Negative	Positive
D29	Small	Round	Entire	Flat	Yellow	Rough	Opaque	Positive	Bacilli	Positive	Positive
D30	Medium	Round	Entire	Concave	White	Smooth	Opaque	Negative	Rods	Negative	Positive
D31	Small	Round	Entire	Concave	Off white	Smooth	Opaque	Negative	Rods	Negative	Negative
D32	Small	Round	Irregular	Concave	Off white	Mucoid	Opaque	Negative	Rods	Negative	Negative

Figure 1. Screening agarolytic activity of isolate D8.

Identification of potential producer isolate of agarase enzyme (D8):

According to the microscopical and morphological characteristics, isolate D8 was revealed to be belonged to the *Bacillus* genus. Based on guidelines established in Bergey's Manual of Systematic Bacteriology, it can be concluded that isolate D8 was nearly associated with *Bacillus subtilis* according to the findings presented in **(Table 2).**

Table 2. Biochemical tests of potential producer bacterial isolate of agarase enzyme (D8).

Test	Result
Starch hydrolysis	Positive
Voges Proskauer	Positive
Citrate utilization	Positive
Indole production	Negative
Methyl red	Negative
Gelatin liquefication	Positive
Casein hydrolysis	Positive
Lipase production	Negative
Nitrate reduction	Positive
Urease production	Negative
Catalase	Positive
6.5% NaCl growth	Positive
Acid production	
D-Glucose	Positive
L-Arabinose	Positive
D-Xylose	Positive
Trehalose	Positive
D-Mannitol	Positive
Raffinose	Positive
D-Lactose	Negative
D-Fructose	Positive
D-Galactose	Negative
Sorbitol	Positive
Sucrose	Positive
D-Maltose	Positive

Factors impacting Bacillus subtilis growth and agarase production:

The incubation duration, pH, temperature, NaCl content, and different carbon and nitrogen sources were all studied for the growth and agarase production of the isolated bacteria *Bacillus subtilis*.

The incubation period impact:

The incubation period for agarase production was studied as shown in **Figure 2** by *Bacillus subtilis* in the basal salt solution medium B which contains 0.2% (w/v) agar as substrate. During the culture's growth, the agarase activity increased and the maximum time for the production of the enzyme was obtained after 2 days with activity (2.055 U/ml±0.006). After 3 days, there was a significant decrease ($p<0.05$) in agarase enzyme production. The minimum level for the production of agarase was obtained after 5 days with activity (0.718 U/ml±0,018). The maximum growth was measured after 2 days (0.523±0.006) and after three days (0.383±0.007) to five days (0.179±0,003), the growth dramatically decreased ($p<0.05$) to the minimum level.

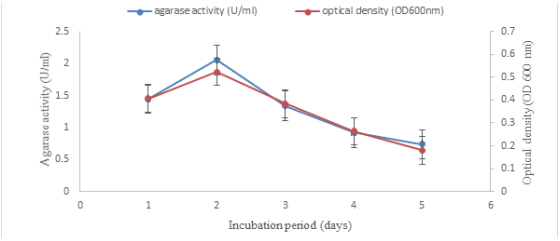

Figure 2. The impact of incubation periods on Bacillus subtilis growth and agarase production. A mean (n = 3) ±SE is used to express data.

The temperature impact:

As shown in **Figure 3,** agarase production by *Bacillus subtilis* was highly influenced by increasing the temperature from 25℃ to 40℃. The maximum production for the agarase enzyme was obtained at 40℃ with activity (2.02 U/ml±0.023). There was a significant decrease ($p<0.05$) in the activity at 45℃, and there was no activity at 55℃. The optimum growth range of *Bacillus subtilis* was from (30℃ - 40℃). The culture growth was zero at 55℃.

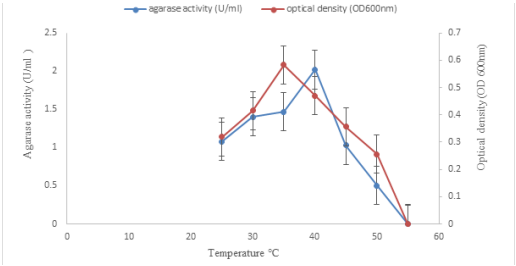

Figure 3. The impact of temperatures on *Bacillus subtilis* growth and agarase production. A mean (n = 3) ±SE is used to express data.

The pH impact:

As demonstrated in **Figure 4**, the best pH for agarase production was 8 with *Bacillus subtilis* activity (2.01 U/ml±0.006). At pH 11, there was a substantial drop ($p<0.05$) in agarase enzyme activity, while at pH 12, there was no activity. The optimum growth range of *Bacillus subtilis* was assessed at pH 6 to pH 10.

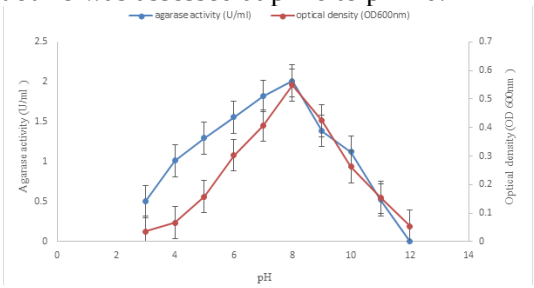

Figure 4. The impact of pH on Bacillus subtilis growth and agarase production. A mean (n = 3) ±SE is used to express data.

The NaCl concentration impact:

As shown in **Figure 5,** 3% NaCl was found to be the maximum concentration for the production of agarase by *Bacillus subtilis* with activity (2.06 U/ml±0.036). There was a significant decrease ($p<0.05$) in agarase activity until reached the minimum level of production at 5% with activity (0.509 U/ml±0.001). The optimum range of NaCl concentration for maximum growth of *Bacillus subtilis* was assessed at 2% to 3.5%.

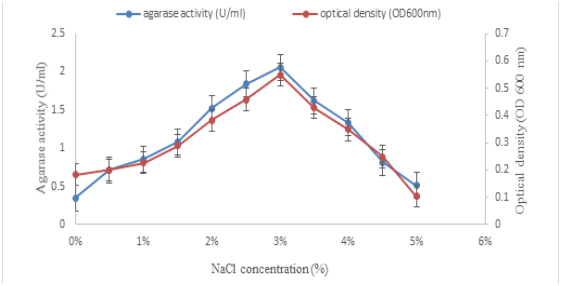

Figure 5. The impact of various NaCl concentrations on Bacillus subtilis growth and agarase production. A mean (n = 3) ±SE is used to express data.

The various carbon sources impact:

As shown in **Figure 6,** different carbon source's effect on the growth of *Bacillus subtilis* and agarase production was studied. Agar was found to be the optimum carbon source for agarase production by *Bacillus subtilis* with activity (2.1 U/ml±0.038) and the optimum growth was also obtained with agar (0.583±0.002). Agarose, galactose, glucose, fructose, sucrose, lactose, and maltose were considered to be good sources of carbon for the production of agarase enzyme but using these carbon sources made the agarase activity decline significantly ($p<0.05$). Little bacterial growth was noticed with trehalose, xylose, and xylan. The best agar concentration for maximum production of agarase was 0.4 % with activity (2.04 U/ml±0.015). There was a significant decrease ($p<0.05$) in agarase activity when the concentration of agar increased. However, the optimum *Bacillus subtilis* growth occurred at 0.3 % agar concentration (0.555±0.016) as shown in **Figure 7**.

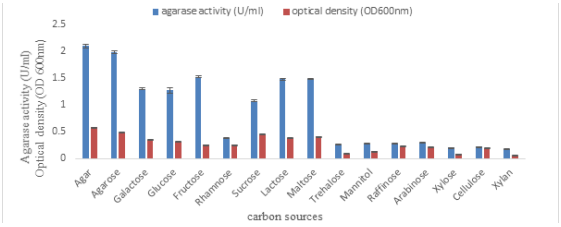

Figure 6. The impact of various carbon sources Bacillus subtilis growth and agarase production. A mean (n = 3) ±SE is used to express data.

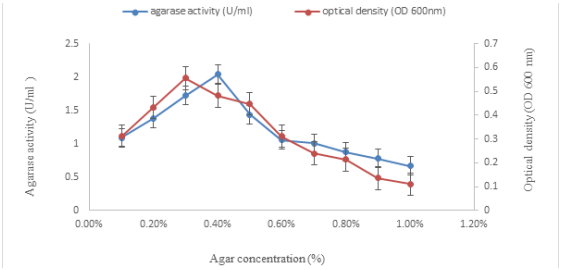

Figure 7. The impact of various agar concentrations on Bacillus subtilis growth and agarase production. A mean (n = 3) ±SE is used to express data.

The various nitrogen sources impact:

As shown in **Figure 8,** different nitrogen source's effect on the growth of *Bacillus subtilis* and agarase production was studied. Yeast extract was found to be the optimum nitrogen source for agarase production by *Bacillus subtilis* with activity (2.13 U/ml±0.043) and the optimum *Bacillus subtilis* growth was also obtained with yeast extract (0.579±0.004). Little activity of agarase was noticed with glycine and casein. Little bacterial growth was noticed with glycine (0.105±0.003). As shown in **Figure 9**, the best yeast extract concentration for maximum production of agarase was 0.3 % with activity (1.93 U/ml±0.041). There was a significant decrease ($p<0.05$) in agarase activity when the concentration of yeast extract increased. However, optimum *Bacillus subtilis* growth occurred at 0.4 % yeast extract concentration (0.557±0.006).

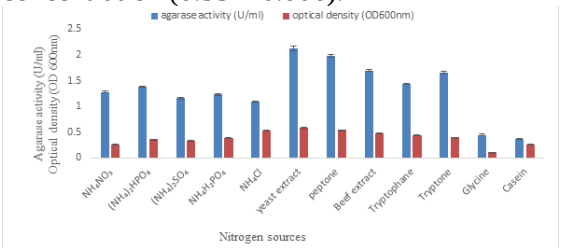

Figure 8. The impact of various nitrogen sources on Bacillus subtilis growth and agarase production. A mean (n = 3) ±SE is used to express data.

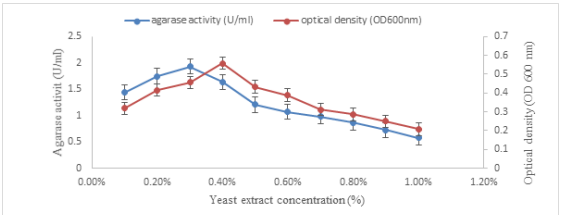

Figure 9. The impact of various yeast extract concentrations on Bacillus subtilis growth and agarase production. A mean (n = 3) ±SE is used to express data.

Discussion:

Massive natural resources with excellent biological activities can be found in marine areas. Degradative enzymes are among the unique biomolecules that marine bacteria can produce (Jayasree et al., 2021). The objective of the current investigation is to isolate marine bacteria from the coast of the Mediterranean Sea of New Damietta City Governorate, Egypt, and screen their ability to produce agarase enzyme and to optimize medium conditions for increasing the activity production of agarase in free cell cultures of *Bacillus subtilis.* The results showed that among 32 bacterial isolates, 12 bacterial isolates produced agarase enzyme. The isolate designated as D8 was found to be the higher producer isolate of agarase enzyme. Isolate D8 was identified as *Bacillus subtilis* based on its microscopical, morphological, and biochemical properties. Agarase belongs to the glycoside hydrolase (GH) enzyme family, which allows bacteria to use agar as a carbon and energy source (Pluvinage et al., 2013). Most agarolytic bacteria originate from aquatic, particularly marine environments. The characteristics and relative quantities of agarase enzymes generated by agar hydrolyzing bacteria determine how effectively agar is hydrolyzed. The agar degradation was detected by the solution of iodine. The agarase enzyme production by isolated bacteria resulted in the development of a clear yellow zone around the agarolytic bacterial colonies, which indicated effective degradation of the agar polysaccharide structure. In contrast, the non-degraded agar portions were dark in brown color (Furusawa et al., 2017). The fermentation process needs to take place in ideal conditions. To achieve the optimal conditions, various optimization techniques must be used. One of those preliminary optimization techniques is the conventional optimization technique known as the One Factor At-Time (OFAT) method. Major variables including temperature, fermentation time, production medium pH, NaCl concentration, and various sources of carbon and nitrogen were sequentially adjusted in this method. The growth and agarase production by Bacillus subtilis increased gradually and reached the maximum values after 2 days. Park et al. (2014) and Labade et al. (2023b) were obtained similar findings. The activity of the enzymes is thought to be greatly influenced by temperature. The ideal agarase production temperature is about 38°C according to Fu & Kim, 2010. While in our study, 40°C was the ideal temperature for agarase production by Bacillus subtilis which was agreed with the findings noted by Roseline & Sachindra (2018), Sharabash et al. (2022), and Veerakumar & Manian (2022). Due to thick bundles of gelled agar that can limit enzyme activity, the maximum operating temperature of different agarases is higher than the temperature at which agar gels (Feng & Li, 2013). At higher temperatures, agarase activity is lost due to denaturation of the enzyme, which impacts the development of the complex of enzyme and substrate. The ionic state of the agarase is influenced by pH, which in turn changes the tertiary structure and activity of the enzyme, making the enzyme highly dependent on pH. Additionally, pH changes the active sites and results in permanent inactivation. Enzyme denaturation and sharp drops in enzyme activity can result from any deviation from the ideal pH. The majority of known marine agarases perform effectively at neutral (Wang et al., 2006) or weakly alkaline pH (Fu et al., 2008), which is consistent with seawater's naturally weakly alkaline pH (pH 7.5) (Long et al., 2010). The ideal pH for Bacillus subtilis production of extracellular agarase was 8. The same results were noted by Shi et al. (2008), Dong et al. (2021), and Labade et al. (2023a). Osmotic pressure formed in the microbial environment is usually the cause of cell lysis upon the addition of NaCl at a greater concentration. Therefore, it would be required to determine the impact of various NaCl concentrations between 0 and 5% (w/v) with production interval of 0.5% on the culture of the growth and activity of the enzyme. It was found that a concentration of 3% NaCl was ideal for Bacillus subtilis to produce agarase enzyme. A similar observed results with an optimum enzyme activity at 3% NaCl were stated by Sorkhoh et al., 2010 and Lee &

Lee, 2014. Agar was found to be the most effective carbon source for Bacillus subtilis to produce agarase. It is well-established that various bacterial strains can produce agarase when exposed to agar (Saraswathi et al., 2011; Ziayoddin et al., 2014; Rajeswari et al., 2016; Cheba, 2022). The amount of agarase production was decreased when we used another carbohydrate as a carbon source. It is well known that the highest conversion efficiency and best substrate utilization occur in any fermentation process when the correct substrate concentration is present. It was discovered that variations in the agar concentration affected the amount of agarase produced. Enzyme activity was measured with 0.1–1% (w/v) agar amount. Agarase production by Bacillus subtilis was found to be highest with 0.4% (w/v) agar, and when agar concentration was increased over 0.4%, agarase production was decreased. This may be due to the increasing of the agar concentration made the culture medium more viscous, which impeded the growth of cells and reduced the agarase production (Fu et al., 2009). According to Roseline & Sachindr (2016), the optimal agar concentration was between 0.1 and 0.5%. Nitrogen sources are essential as basic materials for bacteria to generate protein and nucleic acid (You et al., 2023). Enzyme production and culture growth were positively impacted by the organic nitrogen sources. Using yeast extract as a nitrogen source led to the maximum agarase production and Bacillus subtilis growth. These results were like the results detected by Lakshmikanth et al. (2006), Saraswathi et al. (2011), and Roseline & Sachindra (2018). The maximum amount of agarase production by Bacillus subtilis was obtained with 0.3% (w/v) yeast extract, and when the yeast extract concentration was increased, the amount of agarase produced decreased. This observation is similar to (Fu et al., 2009). Due to the high peptide and amino acid content of yeast extract, the increasing concentration had a suppressive impact on the production of enzymes (Mehta et al., 2006).

Conclusion:

The isolate producing higher agarase activity was *Bacillus subtilis* which was identified according to its morphological and biochemical characteristics. The ideal conditions for *Bacillus subtilis* to produce the agarase enzyme were investigated. The optimum incubation period was 2 days. The best temperature was 40°C. The preferred pH value was 8. The recommended NaCl content was 3%. The optimum carbon source was agar with a concentration of 0.4%. The optimum nitrogen source was yeast extract with a concentration of 0.3%. This provides the ideal medium composition for higher production of agarase enzyme by this bacterium.

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الملخص العربي

عنوان البحث: عزل وتحسين انزيم الأجاريز بواسطة البكتيريا البحرية باسيلس ساتلس

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تم عزل اثنان وثلاثون مستعمرة بكتيرية مختلفة شكليا من ساحل البحر المتوسط بمدينة دمياط الجديدة، مصر بواسطة تنقية الإستزراع علي وسط الأجار المغذي الذائب في مياه البحر. تم اختبار كل العزلات لنشاط الأجاريز علي وسط محلول ملح قاعدي ب. تم استخدام محلول لوغول اليود لتتبع نشاط الأجاريز. من بين اثنان وثلاثون عزلة، أظهرت اثنا عشر عزلة نشاط الأجاريز. تم قياس نشاط الأجاريز بواسطة طريقة داي نيترو سالسيلك أسيد. سجلت العزلة (D8) الحد الأقصى للنشاط التحللي للأجار. تم تأكيد تعريف العزلة المحللة للأجار بناء علي الإختبارات المجهرية والشكلية والكيميائية الحيوية. تم تعريف البكتيريا علي أنها باسيلس ساتلس. تم تحسين ظروف الوسط لتحقيق أقصي نمو لباسيلس ساتلس وإنتاج الأجاريز جارج الخلية. كان وقت التخمر المثالي، درجة الحرارة، الرقم الهيدروجيني، وتركيز كلوريد الصوديوم التي تحتاجها باسيلس ساتلس لتنتج النشاط الأمثل للأجاريز هو٤٨ ساعة، ٤٠ درجة مئوية، ٨، و ٠,٣ في المائة علي التوالي. وجد أن مصدر الكربون الأمثل لإنتاج الأجاريز هو الأجار (٠,٤ في المائة) من بين الكربوهيدرات التي تم اختبارها. أفضل مصدر للنيتروجين لانتاج الباسيلس ساتلس الأجاريز هو مستخلص الخميرة (٠,٣ في المائة).