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NEW METHOD FOR DETERMINATION OF AGARASE ENZYME FROM BACTERIAL ORIGIN

(With 5 Figures)

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طريقة جديدة لتقدير نشاط إنزيم الاجاريز من أصل بكتيري

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يهدف هذا البحث على استنباط طريقة جديدة لتقدير نشاط إنزيم الاجاريز من أصل بكتيري. اشتملت الدراسة على استخدام العزلة البكتيرية *Bacillus macernas* SM. تم دراسة الظروف المثلى لتقدير نشاط الإنزيم فى مخلوط التفاعل وكانت كالتالي: افضل وقت للتحضين كان عند ٤٨ ساعة وانسب درجة حرارة هى ٥٠°م , أنسب تركيز لمادة الصمغ العربي كانت عند ١,٥% وأنسب تركيز لمادة التفاعل كانت عند ١% هذه مثلت أفضل الظروف لنشاط الإنزيمات المختبرة. فى محاولة لتحديد انسب محلول منظم لنشاط إنزيم الاجاريز تم إجراء عدة محاليل كالتالي: المحلول الكابح Tris-HCl وكان انسب pH عند ٧,٢ و المحلول الكابح boric acid borate وكان انسب pH عند ٨,٨ والمحلول الكابح Phosphate وكان انسب pH عند ٦,٨ و المحلول الكابح Citrate phosphate وكان انسب pH عند ٥,٨. تمت الطريقة الجديدة بتجميع كل هذه الظروف المناسبة للحصول على أعلى نشاط من إنزيمات الاجاريز فى مخلوط التفاعل وسوف يتم تطبيقها في مجالات مختلفة وخضوعها للدراسات التطبيقية والصناعية باستخدام تلك الإنزيمات الحالية في عمليات الهندسة الوراثية وفي تكنولوجيا صناعة الغذاء. وبناء على هذا يمكن تطبيق هذه الطريقة على المستوى المعملي والتجريبي أو على المستوى الصناعي والتجاري نظرا لكفاءتها الملحوظة في العمل والنشاط عند درجة الحرارة والقلوية العالية وخصوصا في مجالات الهندسة الوراثية وأشباهها كما يمكن التركيز على إمكانية استخدامها في مجالات الصناعة والغذاء نظرا للنتائج التي تم التوصل إليها في هذا البحث بالإضافة إلى قدرة الإنزيمات الحالية بالعمل والنشاط عند ظروف عالية من الحرارة تصل الى ٥٠ درجة مئوية مما يؤكد أهميتها فى عديد من مجالات التكنولوجيا الحيوية وذلك باستخدام تركيزات مقبولة علميا وعمليا .

SUMMARY

This context concentrated mainly on determination of a new method for agarse enzyme assay by so called agar cup plate clearing zone(ACZ)

technique. *Bacillus macernas* SM produced constitutive extracellular agarase, and could grow without using agar in its culture medium. Optimization of this new technique controlled by using the most important parameters of enzymes assay. An optimum gum arabic concentration was at 1.5%(w/v); optimum substrate (agar) concentration was detected at 1%; the best temperature for enzyme assay in its reaction mixture was detected at 50°C; the best time to get the highest rate of enzyme activity determined after 48h. In attempt to determine the most applicable buffer at its identical pH value to get the highest rate of agarase enzyme activity, different buffer have been carried out; Tris-HCl was represented with optimum pH value of 7.2; boric acid borate buffer (8.8); Phosphate buffer (6.8); Citrate-phosphate buffer (5.0); Citrate buffer (5.8). This technique may be used fairly in molecular microbiology and biotechnology, also from the essential application of this technique for differentiation between enzymes assay produced by the same organism that degrade not only agar but also various plant polysaccharides, i.e., cellulose, pectin, starch, and xylan.

Key words: Agarase determination, New technique, *Bacillus macernas* SM.

INTRODUCTION

Agar is a polysaccharide found in the cell walls of some red algae and is unusual in containing sulfated galactose monomers. It requires nothing but extraction and purification to become agar, but is sometimes chemically modified into agarose for special applications, agar is the queen of gelling agents (Humm, 1947).

Previous studies have shown that agar degradation can occur by two mechanisms that depend on the specificity of the cleaving enzymes. The first pathway for agar breakdown comes from studies on *Pseudoalteromonas atlantica* ATCC 19292 (Morrice, *et.al* 1983a& Morrice, *et.al* 1983b) and relies on extracellular agarases. In this bacterium, an endo -agarase I cleaves the -(1,4) linkages of large agar polymers to a mixture of oligosaccharides with neoagarotetraose as the final product. The second lytic mechanism involves the cleavage of -(1,3) linkages on agarose by extracellular agarases (Potin, *et.al* 1993,Young, *et.al* 1971 and Young, *et.al* 1978), yielding oligosaccharides from the agarobiose series, which contain D-galactose at the non reducing end.

A marine bacterium strain degraded numerous complex carbohydrates, such as agar, chitin and alginate. It may play an important role in altering carbon fluxes in marine environments. End-product analysis revealed that 2-40 synthesized an agarase system that consisted of at least three enzymes, beta-agarase I, beta-agarase II and alpha-agarase, which acted in concert to degrade polymeric agar to D-galactose and 3,6-anhydro-L-galactose, Whitehead *et. al,* (2001).

Agar is important jellifying agent for biochemical use and in food industry. To cleave the beta-1,4 linkages between beta-D-galactose and beta-L-3,6-anhydro-galactose residues in the red algal galactans known as agar, marine bacteria produce polysaccharide hydrolases called beta-agarases. Beta-Agarases A and B from *Zobellia galactanivorans* Dsij have recently been biochemically characterized, Allouch *et. al.* (2003).

MATERIALS and METHODS

Bacterial strain used

The agarolytic features of identified bacteria isolated from polluted *Solanum tuberosum* wastes was investigated. The strain was identified as *Bacillus macernas* SM, in solid agar, this isolate produced a diffusible agarase that caused agar softening around the colonies

Enzyme assay

The New method for determination of agarase enzyme from bacterial origin called agar cup plate clearing zone (A.C.Z.) technique was adopted from the estimation procedure of uric acid clearing zone (UCZ) by Ammar *et.al.* (1986), and pectin clearing zone (PCZ) by Ammar *et.al,* (1995), Afifii (1994) and Afifii (2002).

Optimization of agarase clearing zone (ACZ) technique

This was performed using the crude enzyme preparation of agarase produced by *Bacillus macernas* SM, these factors were carried out in order to optimize agarase assay in its reaction mixture in the assay medium of (ACZ) technique as represented in Ammar *et.al,* (1995) and Afifi (1994).

Gum Arabic concentration

Different concentrations of Gum arabic (%,w/v) were applied viz. 0.25, 0.50, 1.0, 1.5 and 2.. At the end of incubation period agarase activity was determined by the present new assay technique in enzyme reaction mixture.

Incubation time

Bacillus macernas SM agarase was incubated at different periods viz. 6,12,18,24,36,48 and 60 h. at 30°C. At the end of incubation interval, enzyme activities were assayed.

Substrate concentration

Different concentrations of agar (%,w/v) were applied viz. 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00. At the end of incubation period, rate of agarase enzyme catalyzed reaction has been assayed.

Temperature

Bacillus macernas SM agarase was incubated at different temperatures viz. 10,20,30,40,50 and 60°C and finally tested for its activity.

Application of different buffers at their various pH values

Different buffere applied at various pH values prepared as the method of Gomori (1955) as follows: Tris-HCl buffer has applied at pH values 7.2, 7.6, 8.0, 8.6; boric acid borate buffer (7.6, 8.0, 8.8 and 9.0); Phosphate buffer (5.7, 6.1, 6.4, 6.8, 7.2, 7.8 and 8.6); Citrate phosphate buffer (3.4, 4.2, 5.0, 5.8, 6.4 and 7.0) and Citrate buffer (3.6, 4.2, 4.3, 4.8, 5.2, 5.8, 6.2, 6.4 and 7 .0). Each identical reaction mixture of each pH value has inoculated and incubated and then tested for maximum rate of agarase enzyme catalyzed reaction.

RESULTS

The results of this context regarded with the optimization of almost all of essential parameters controlling this new technique of agarase enzyme assay.

Gum arabic concentration

Data illustrated graphically in Fig. (1) revealed that an optimum gum arabic concentration to fulfill the highest agarase enzyme activity was detected at 1.5%. Beyond this optimum concentration, enzyme activity sharply decreased.

Substrate concentration

The best enhanced substrate (agar) concentration to reach the highest level of agarase enzyme could be included in the presence of 1% of agar as illustrated in Fig. (2). Below or above this concentration, enzyme activities were decrease gradually.

Incubation time

It was shown from the data in Fig.(3) that the rate of agarase catalyzed reaction reached its maximum value after 48 hours in its

reaction mixture. Before or after this time, enzyme activity has decreased markedly.

Temperature

Data illustrated in Fig.(4) revealed that the rate of agarase enzyme fulfilled its maximum activity at 50°C in its reaction mixture. Beyond this identical temperature, rate of enzyme markedly decreased.

Application of different buffers

Different buffers have been applied at various pH values as graphically represented in Fig.(5) and revealed that almost all of applied buffers resulted in closely related activities of enzyme used. Whoever, some lower pH values (5.0 and 5.8) revealed 216 units/ml of citrate phosphate buffer,others higher pH values (5.8 and 7.2 revealed 216 and 226 units/ml of citrate and Tris-HCl buffers, and the highest others pH values (6.8 and 8.8 with 220 and 240 units/ml of phosphate and boric acid borate buffer respectively. Below or above these particular pH values, agarase enzyme activities were decreased gradually.

DISCUSSION

This context concentrated mainly on application of a novel technique for agarase enzyme assay by using (agar cup plate clearing zone) technique. A constitutive extra cellular agarase *produced by Bacillus macernas* SM previously isolated from polluted *Solanum tuberosm* wastes in upper Egypt, and could be grown on its culture medium without agar. Optimization of this new technique controlled by using the most important parameters of enzymes assay.

In accordance to our results, Leon, *et.al,* 1992 has worked on a marine bacterial strain isolated from the Bay of San Vicente, Chile, and identified it as *Alteromonas* sp. strain C-1 then declared that this strain produced high levels of an extracellular agarase in the presence of agar, the enzyme hydrolysis agar and yielding neoagarotetraose as the main product, was obtained at an optimum pH of about 6.5.

Also, Hosoda *et.al,* 2003 reported that, Agar-degrading bacteria in spinach plant roots cultivated in five soils were screened, and four strains of *Paenibacillus* sp. were isolated from roots cultivated in three soils. The agar-degrading bacteria accounted for 1.3% to 2.5% of the total bacteria on the roots.

In experiment to determine the best gum Arabic concentration for agar distributions in its reaction mixture, 1.5% was the best concentration. An optimum substrate concentration that fulfilled the

highest yield of agarase was obtained at 1%. In relation to our results, Jorge *et. al.* 1998 reported that the enzyme hydrolyzed 1,4-glycosydic linkages of agar, yielding neoagarotetraose and neoagarohexaose as the main products, and exhibited maximal activity at pH 7.

Also Van-Meulen and Harder, 1975 have declared that the optimal conditions for extracellular agarase activity were pH 6.3 and 30C. When agarose was used as a substrate, an apparent temperature optimum of 35C was found, due to gelling of the substrate during the assay procedure.

Interestingly, the best temperature and incubation time in the reaction mixture to fulfill the highest agarase enzyme were detected at 50°C for 48 hours respectively.

An attempt to test the best pH values of the present agarase enzyme activity, different buffer have been carried out, Tris-HCl was with optimum pH 7.2;boric acid borate buffer 8.8; Phosphate buffer 6.8;Citrate-phosphate buffer 5.0; Citrate buffer 5.8. respectively.

In relation to the present results, Araki *et.al.* 1998, has mentioned that the suitable pH and temperature for the polysaccharide-degrading enzyme reaction were 6.5 and 22 °C, respectively. Also, Shieh and Jean 1998 have used *Alterococcus agarolyticus*, gen.nov., sp.nov., a halophilic thermophilic bacterium in agar degradation on agar medium, and reported that this strain yielded reducing sugars and organic acids as the end products under either aerobic or anaerobic conditions. The growth temperature range was approximately 38-58°C with an optimal temperature of about 48°C. The five strains tolerated a relatively narrow pH range from 7.0 to 8.5.

This technique may be used fairly in molecular microbiology and biotechnology, also from the essential application of this technique for differentiation between enzymes assay produced by the same organism that degrades not only agar but also various plant polysaccharides, i.e., cellulose, pectin, starch, and xylan.

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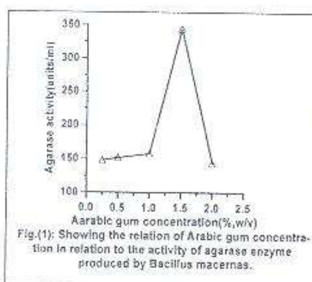

Fig.(1): Showing the relation of Arabic gum concentration in relation to the activity of agarase enzyme produced by Bacillus macernas.

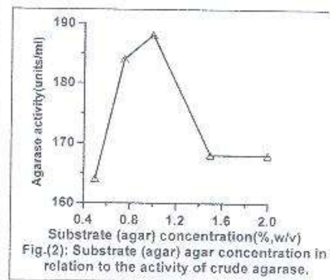

Fig.(2): Substrate (agar) agar concentration in relation to the activity of crude agarase.

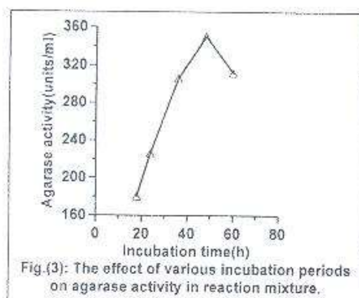

Fig.(3): The effect of various incubation periods on agarase activity in reaction mixture.

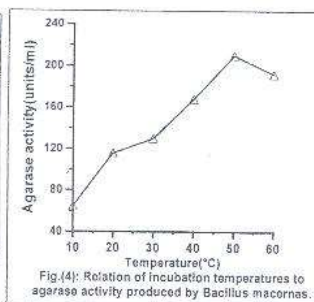

Fig.(4): Relation of incubation temperatures to agarase activity produced by Bacillus macornas.

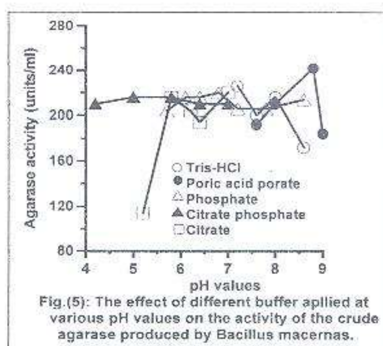

Fig.(5): The effect of different buffer apllied at various pH values on the activity of the crude agarase produced by Bacillus macernas.