Immobilization of *Vibrio cholerae* S1 (NAG) L-Glutaminase on Different Supports

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Abstract:

Sixteen *Vibrio* isolates producing L-glutaminase were obtained from clinical and water samples , one isolate was selected according to its' highest enzyme productivity , it was identified as *Vibrio cholerae* (NAG) and coded as *V. cholerae* S_1.

The bacteria was cultured in a liquid medium (containing L-glutamine) , L-glutaminase was extracted from the cells by ultrasonication , the enzyme was precipitated by 30 % saturation of ammonium sulphate , dialyzed and immobilized by adsorption on different supports including Sephadex G-100 , cellulose powder , starch , silica gel , glass beads and charcoal.

Sephadex G-100 retained most of enzyme activity (90 %) followed by starch (78 %) , then silica gel and cellulose powder (71 %) while glass beads and charcoal retained 58 % only.

The immobilized enzyme was subjected to different temperatures and pHs. The results showed that the immobilized enzyme is more stable than the free enzyme in different temperatures and pHs. Silica gel was the best matrix for protecting L-glutaminase against heat , it retained 52 and 22 % of the original activity after 2 hrs of incubation at 50 and 60 ◌̊c respectively while the free enzyme retained 30 and 10 % at the same conditions.

The immobilized enzyme was more stable at pH 7 than at pH 4 or 10 . the enzyme adsorbed on Sephadex G-100 retained the maximum activity (98 %) at pH 7 for 2 hrs , while it was 73 % for the free enzyme.

The immobilized L-glutaminase of *V. cholerae* S_1 (with Sephadex G-100) was stored at 4 ◌̊c for 30 days , the remaining activity was 35 % , while it was 18 % for the free enzyme.

It can be concluded from these results that *V. cholerae* S_1 L-glutaminase can be immobilized on different inert materials, Sephadex G-100 is more suitable in this project , Silica gel can protect the enzyme against heat. In general the immobilized enzyme is more stable at different temperatures , pH and time than the free enzyme.

Key wards: *Vibrio cholerae* , L-glutaminase, Sephadex G-100, immobilization

Introduction :

Glutaminase (Ec. 3.5.1.2. L-glutamine amidohydrolase) catalyses the reaction L- glutamine + H_2O ⟶L-glutamate + ammonia. It is found in many eucaryotic and prokaryotic cells , it has many advantageous in food and pharmaceutical applications , L-glutaminase is used in cancer treatment[1,2]. *Vibrio* glutaminase has antileukemia effect[3,4].

Glutaminase can be used in food industries as a flavor enhancer , *Vibrio casticola* and *Pseudomonas fluorescens* glutaminase may be added to some kinds of food to give a popular flavor [3].

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Glutaminase is used in oral dehydration solution since it transforms glutamine to glutamate which help in absorption of ions in small intestine [5].

The attachment of enzymes to inert materials is a kind of immobilization methods , it is practical and suitable method for many industrial applications , there are many advantageous for the immobilization of cells and enzymes , such as isolation and purification of enzyme is obviated , reduces the expense of the free enzyme since the immobilized enzyme can be used for many reaction cycles , increasing the stability of enzyme toward the environmental conditions such as pH , temperatures , and ionic strength , reduces the volume of bioreactors and other benefits [6].

The aim of this study is to determine the suitability of different material to immobilize *V. cholerae* L-glutaminase and the stability of the immobilized enzyme at different conditions, which may give many practical and economic advantageous.

Materials and Methods:

Isolation of *Vibrio cholerae* :

Many samples of water and diarrheial stools were collected from different sources, one loop full of each sample was cultured in alkaline peptone water and incubated at 37 ◌̊c for 18 hrs, apart of this culture (one loop full) was cultured on TCBS medium by streaking on the surface and incubated at 37 ◌̊c for 24 hrs.

The yellow colonies were picked and cultured on nutrient agar plates, this step was repeated 2-3 times to obtain pure culture [7]. The pure cultures were preserved on nutrient agar slants at 4 ◌̊c and in nutrient broth with 15 % glycerol at -20 ◌̊c.

Bacterial isolates were identified depending on their morphological , biochemical and serological tests [7,8].

Screening of bacteria on glutamate agar :

Bacterial isolates were cultured on nutrient agar and incubated at 37 ◌̊c for 24 hrs , one colony was inoculated in the center of glutamine agar plate prepared by dissolving 2 gm of L-glutamine , 0.5 g of $MgSO_4$-$7H_2O$, 1 gm of KH_2PO_4 and 20 gm of agar in 1 liter of distilled water , phenol red solution was added (few drops) after adjusting the pH to 6 – 8 and autoclaved at 121 ◌̊c for 15 min.

The pink hallow around the growing colonies was observed and its diameter was measured. The diameter of the pink hallow indicates the efficiency of the bacterial isolates to produce L-glutaminase which liberates ammonia from glutamine resulting in changes of pH to alkaline.

Growing of bacteria in L-glutaminase production medium :

Bacterial isolates were activated in tryptic soy broth for 18 hrs , 2 mls of this culture was added to 100 mls of glutaminase liquid medium [9] and incubated at 37 ◌̊c for 24 hrs in a shaking incubator at 100 rpm.

Assay of glutaminase activity :

Bacterial cells were precipitated by centrifugation at 6000 rpm for 15 min., the precipitate was collected and washed twice with 0.02 M phosphate buffer pH 8.

Fifty microliters of cell suspension was added to 200 microliters of 0.1 M L-glutamine dissolved in 0.02 M phosphate solution pH 8 and incubated at 37 ◌̊c for 30 min in a shaking water bath , the reaction was stopped and glutaminase activity was measured as described by Novack and Philips [10] depending on ammonium standard curve.

One unit of enzyme activity is the amount of enzyme required for production of one micromole of

ammonia from L-glutamine in one minutes under the reaction conditions.

Extraction of L-glutaminase from bacterial cells :

Bacterial cells were harvested from the production medium and washed as described above , then they were disintegrated by ultrasonication (Soniprep 150 sonicator) for 5 min(intermittent). under cold conditions [11]. The solution was centrifuged at 8000 rpm for 30 min., glutaminase activity was measured in the supernatant.

Precipitation of glutaminase :

L-glutaminase was precipitated by 30 % saturation of ammonium sulphate under cold conditions and dialyzed against d. w. for 24 hrs with several charges of water, the activity of L-glutaminase was measured.

Immobilization of the enzyme :

Different solid supports were used for immobilization of L-glutaminase included: charcoal, Silica gel, cellulose powder, glass beads, maize starch and Sephadex G-100. These materials were autoclaved at 121 ◌̊c for 30 min. and dried at 90 ◌̊c in the oven for 24 hrs.

Glutaminase solution was added to these materials at 1: 1 V: W, mixed and incubated for 48 hrs with agitation, the suspensions were filtered by Whatman no. 1 paper, the wet material on the filter paper was washed several times with 0.02 phosphate buffer to remove the unbounded enzyme.

Assay of immobilized enzyme activity :

A quantity of 0.2 gm of the immobilized enzyme was placed in a small clean beaker, 5 ml of reaction solution (L-glutamine solution) was added, incubated at 37 ◌̊c for one hour in a shaking water bath and enzyme activity was assayed after different of the suspension.

Determination of temperature effect on immobilized enzyme :

The immobilized enzyme was incubated at 37, 50 and 60 ◌̊c for 30, 60, 90 and 120 min. , after which it was transferred to ice bath filtered and the activity of L-glutaminase was determined. The free enzyme was treated in the same manner.

Determination of pH effect on immobilized enzyme :

Different buffers with certain pHs were added to the immobilized enzyme at 1 : 1 (w : vol) , acetate buffer at pH4 , phosphate buffer at pH7 and glycine buffer at pH 10 were used for this purpose , the suspensions were incubated at 37 ◌̊c for 30 , 60 , 90 and 120 min after which they were transferred to ice bath , filtered and glutaminase activity was assayed .

Determination of Stability of glutaminase :

The free and immobilized glutaminase (bounded with Sephadex G-100) was stored at 4 ◌̊c for 10 , 20 and 30 days , enzyme activity was determined after each period.

Results & Discussion:

Isolation and identification of glutaminase producing bacteria: sixteen *Vibrio* isolates capable of producing glutaminase were obtained from the different samples, these isolates changed the yellow color of glutamine agar to pink which indicates that they are active in production of glutaminase.

These isolates were cultured in glutamine liquid medium and screening for their glutaminase production. One isolate was selected according to its' higher enzyme activity than the others, it was identified as *Vibrio cholerae* (NAG) and coded as *V. cholerae* S_1.

Many kinds of bacteria and fungi can produce L-glutaminase which may be intracellular or extracellular. glutaminase has an important role in cell metabolism , it hydrolyzes L-

glutamine and provides nitrogenous compounds for cell metabolism [12,13].

Glutaminase found in most members of Enterobacteriaceae , *Escherichia coli* may produce 2 kinds of glutaminase (isozymes) differ in their pH activity and stage of growth formation [14].

Immobilized glutaminase :

Vibrio cholerae S_1 L-glutaminase was adsorbed on different solid materials, Sephadex G-100 retained the maximum enzyme activity which was 90 % followed by starch (78 %) , Silica gel and cellulose retained 71 % while charcoal and glass beads retained 58 % only (figure 1).

The high remaining activity for Sephadex G-100 immobilized enzyme may be attributed to its porous structure which provides large surface area for adsorption of enzyme particles.

Covalent attachment of dimeric form of renal glutaminase to CNBr-activated Sepharose was achieved with 84 % retention of activity [15].

Microbial transglutaminase was immobilized on controlled – pore glass beads (CPG – 3000) and used for treating whey proteins [16].

The effect of cellulose, chitin and silicate on *Vibrio* adherence was studied, it was found that chitin is the best surface of bacterial adherence [17]. *V. cholerae* can adsorbed on starch granules and utilize it as carbon source this may lead to eliminate the bacteria from intestine [18].

L-glutaminase of marine fungus *Beauveria bassiana* was immobilized by entrapment in Ca-alginate and used in a packed bed – reactor – parameters influencing bead production and performance under batch mode were optimized [19].

Effect of temperature on immobilized L-glutaminase :

Vibrio cholerae S_1 L-glutaminase immobilized with Sephadex G-100, Silica gel and charcoal were selected to study the influence of temperature and pH on it since they represent different chemical compound, Sephadex G-100 is an organic polymer (polysaccharide), Silica gel is inorganic inert material and charcoal is a carbonaceous material.

Glutaminase immobilized with Sephadex G-100 retained 84 % of the original activity when it was incubated at 37 ◌̊c for 120 min. comparing with 75 % for the free enzyme and 78 % for each of silica gel and charcoal (figure 2).

The activity of glutaminase decreased at 50 ◌̊c and 60c , it was observed that the immobilized enzyme (with the 3 kinds of supports) is more stable than the free enzyme in all temperatures used in this study (figure 2 a , b , c) .Silica gel was the best matrix for protecting glutaminase against heat , it retained 54 and 22 % of the activity for 24 hrs at 50 and 60 ◌̊c respectively followed by charcoal which retained 50 and 18 % respectively while the remaining activity for Sephadex G-100 was 42 % at 50 ◌̊c and 18 % at 60 ◌̊c compared with 30 and 10 % for the free enzyme at the same conditions.

It can be concluded from these results that L-glutaminase of *V. cholerae* S_1 is heat sensitive but when it was immobilized it became more resistant, this point has many advantages in industrial applications when there is a need to use high temperatures.

The immobilized transglutaminase extracted from microbial cells lost about 70 % of its activity at 40 ◌̊c after circulation for 8 hrs in a bioreactor [16] , Glutaminase of *E. coli*

immobilized in a hydrogel matrix stay active at 40 – 60 ◌̊c [20].

Vibrio strain Ak-1 grow at 25 ◌̊c adhered avidly to Sepharose beads containing covalently bound B-P-galactopyranoside but failed to bind at 16 ◌̊c [21].

Effect of pH on immobilized glutamines :

The immobilized glutaminase was more stable at pH 7 than at pH4 or 10 (figure 3).

Sephadex G-100 retained 98 % of enzyme activity, silica gel and charcoal retained 77 % while the free enzyme retained 73 % after 2 hr of incubation at pH7.

Sephadex G-100 stabilized L-glutaminase more than silica gel and charcoal at pH4 and 10 , However the remaining activity at acidic (pH4) or basic (pH10) environment was low , which indicates that *V. cholerae* S_1 glutaminase is sensitive to pH changes and it favors the neutral environment.

In an experimental and theoretical studies of reduction diffusion model of two immobilized enzymes participating in the cellular acid – base metabolism namely glutaminase and urease, they were unstable at pH 6, where any perturbation will drive the system towards a more alkaline or more acidic pH owing to the autocatalytic behavior with response to pH exhibited by both enzymes, the optimum pH for the immobilized enzymes was 5.1 [22].

The immobilized L- glutaminase of *Beauveria bassiana* was used in a packed – bed reactor, it was found that the suitable pH of the medium is pH 9 [19].

Stability of immobilized L-glutaminase :

The Sephadex G-100 immobilized enzyme was stored at 4 ◌̊c for 10 – 30 days to determine the stability of *V. cholerae* S_1 L- glutaminase, the activity of free and immobilized enzyme declined with time but the immobilized enzyme retained more activity than the free enzyme after 30 days the remaining activity of the immobilized enzyme was approximately twice of the free enzyme (figure 4). The immobilized enzyme lost 50 % of the original activity after 20 days and 65 % after 30 days while the free enzyme lost 64 and 82 % respectively.

High stability of enzyme activity is an important feature recommended in most application such as enzyme bioreactor used in industrial production.

The immobilized L- glutaminase of *E. coli* remain stable for 24 days compared with 8 days for native enzyme [20].

The immobilized glutamate oxidase and catalase in a polycarbonate membrane showed good operational stability (at least 5 days) , this membrane are suitable for monitoring glutaminase when used in a glutamine biosensors for biotechnological application [23].

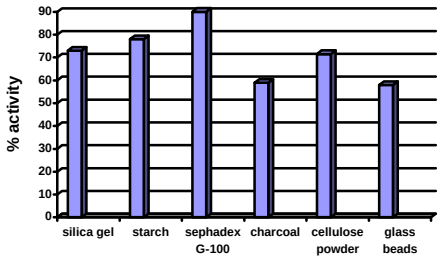

Figure (1) : the remaining activity (%) of *V. cholerae* S1 L-glutaminase immobilized on different matrices.

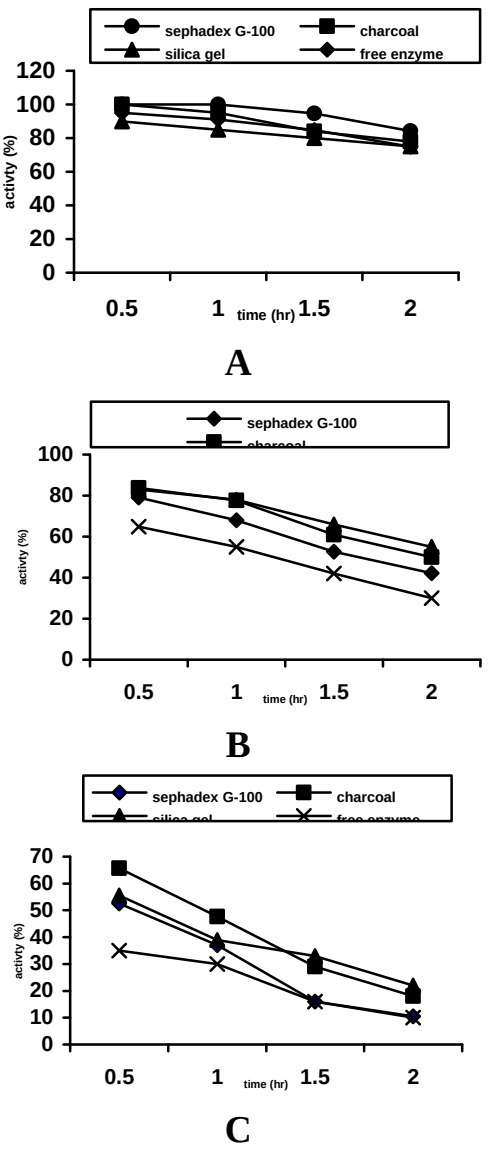

Figure 2 : Effect of temperature on immobilized L-glutaminase
a = 37 ◌̊c , b = 50 ◌̊c , c = 60 ◌̊c

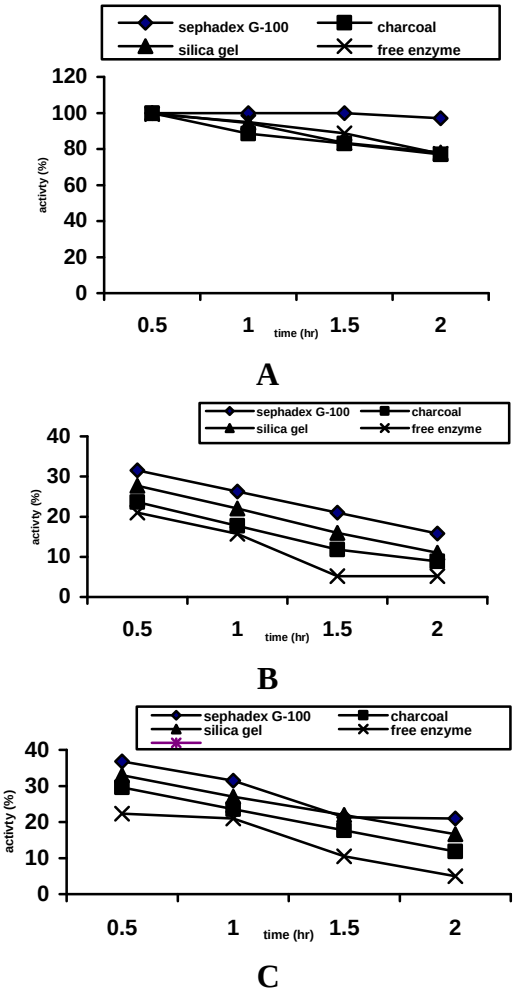

Figure (3) : Effect of pH on *V. cholerae* S1 L-glutaminase immobilized with different materials
a= 7 , b=4 , c=10

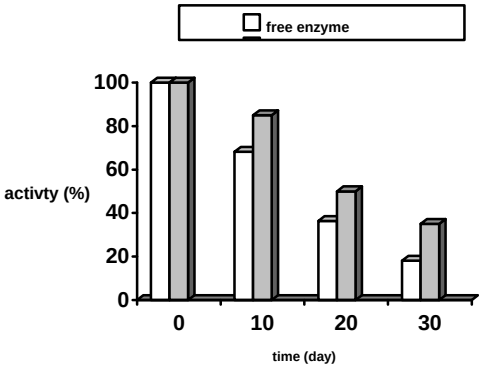

Figure (4) : Stability of *V.cholerae* S1 L-glutaminase storaged at 4 ◌̊c

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تقييد انزيم الكلوتامينيز المستخلص من *Vibrio cholerae* S_1 بامتزازه على مواد ساندة مختلفة.

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الخلاصة :

تـــم الحصـــول عـــلـــى ست عشـرة عزلـة لبكتريـا *Vibrio* منتجـة لانـزيم الكلوتـامينيز (L-glutaminase) من نمـاذج سـريرية ومياه ، وانتخبت عزلـة واحـدة اعتمـاداً علـى انتاجيتهـا الاعلـى للانـزيم ، وشخصت على انها *Vibrio cholerae* (NAG) واعطي لها الرمز S_1.

نميت البكتريـا فـي وسـط سـائل (يحتـوي علـى الكلوتـامين) ، واسـتخلص الانـزيم مـن الخلايـا بعـد تكسـيرها بالموجات فوق الصوتية.

ورسب الانزيم باستعمال كبريتات الامونيوم بنسبة اشباع 30% ، وتمت ديلزته ، ثم قيد بـامتزازه علـى مواد سـاندة مختلفـة تضـمنت السـيفادكس (Sephadex G-100) ، ومسـحوق السـليلوز ، والنشـا ، وهـلام السـليكا ، والخرز الزجاجية ، والفحم.

بينت النتائج ان Sephadex G-100 يحتفظ بمعظم فعالية الانزيم (90 %) يعقبه النشا (78 %) ثم هلام السليكا والسليلوز (71 %) بينما أحتفظ الفحم والخرز الزجاجية بـ 58 % فقط من فعالية الانزيم.

تم تعريض الانزيم المقيد والحر لدرجات حرارية وقيم رقم هيدروجيني مختلفة ، ولـوحظ ان الانـزيم المقيد اكثر ثباتاً من الانزيم الحر في درجات الحرارة المختلفة وقيم الرقم الهايدروجيني ، واظهر هلام السليكا حماية جيدة للانزيم في درجات الحرارة العالية إذ بقي محتفظاً بـ 54 % و 22 % من الفعالية الاصلية عند تعريضه لدرجة 50 و 60م لمدة ساعتين على التوالي في حين احتفظ الانزيم الحر بـ 30 % و 10 % في هاتين الدرجتين.

لـوحظ ان الانـزيم المقيـد اكثـر ثباتـاً بـرقم هايـدروجيني 7 ممـا عليـه بـرقم 4 او 10 واحـتفظ الانـزيم المقيـد بالسيفادكس (Sephadex G-100) باعلى نسبة فعالية (98 %) عند الرقم الهايدروجيني 7 لمدة ساعتين بينما احتفظ الانزيم الحر بـ 73 % في الظروف نفسها. وعندما حفظ الانزيم المقيد بدرجة 4م لمدة شهر بقي محتفظاً بـ 35 % من فعاليته قياساً بالانزيم الحر الذي احتفظ بـ 18 %.

من هذه النتائج يتبين ان انزيم الكلوتامينيز المنتج من بكتريا *V. cholerae* S_1 يمكن تقييده بطريقة الامتزاز على مواد مختلفة ، وان Sephadex G-100 مادة ملائمة لتقييده والحفاظ على فعالية عالية ، وان هلام السليكا يحمي الانزيم من الحرارة العالية بشكل افضل من غيره والانـزيم المقيد عموماً اكثر ثباتاً من الانـزيم الحـر فـي درجات الحرارة وقيم الرقم الهيدروجيني المختلفة فضلاً عن ثباته الخزني.