Gum Tragacanth Gels as a New Supporting Matrix for Immobilization of Whole-Cell

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ABSTRACT: *We introduce a new smooth, non-toxic, biocompatible method for cross-linking of gum tragacanth (GT), a polysaccharide of natural origin, in order to serve as a new supporting matrix for immobilization systems. The modified gum is used as a matrix for the catalysis of the conversion of benzyl penicillin to 6-aminopenicillanic acid (6-APA) by means of Escherichia coli ATCC11105 with penicillin G acylase (PGA) activity. The results show that GT beads can not only serve as a proper matrix for immobilization, but show enhanced hydrolysis rate and stability compared to other immobilization systems used for this reaction. This signifies the potential of GT as a biocompatible matrix for immobilization and its positive prospects for use in more demanding immobilization applications where traditional matrices such as alginate may fall short. The effect of environmental factors, such as temperature, pH, and substrate concentration, have also been studied on the hydrolysis rate and compared with the other immobilizing systems used for the same reaction, such as calcium alginate. Under the optimal conditions, penicillin G conversion reached 91.5% after 6 h and remained over 80% after 45 repeated cycles of 6 h each.*

KEY WORDS: *Gum tragacanth, Immobilized whole-cell, Penicillin G acylase, Ionomer, Crosslink.*

INTRODUCTION

Gum Tragacanth [CAS 9000-65-1] is widely used as a natural emulsifier and thickener in the food, drug, and allied industries. Its wide use is due to its stability in a wide range of temperature and pH, and its effectiveness as an emulsifying agent with extremely long shelf life. The high viscosity imparted to water by GT makes it useful for preparing aqueous suspensions of insoluble substances [1 , 2]. As GT is a natural polymer, it is non-toxic and biocompatible, making it a suitable medium for cell growth. Though its use as an immobilization matrix, to the best of our knowledge, is unprecedented, it has been used in other cell growth applications. *Dobos* used

GT for Plaque assay for animal viruses [3]. The heat-induced cell damage in this assay can be avoided by the use of GT. *Kedmi* and *Katazenelson* used GT for fluorescent antibody assay [4]. Unlike other gums, GT does not prevent the adhesion of suspended cells to glass to form a confluent monolayer due to increasing the viscosity of culture medium.

When GT is mixed with water, only the soluble fraction, called *tragacanthin*, dissolves to give a colloid hydrosol whereas the insoluble fraction, consisting of 60-70% *bassorin*, swells to a gel. Chemically, tragacanthin is a complex mixture of acidic polysaccharides containing D-galacturonic acid. The other sugars produced on hydrolysis are D-galactose, D-fructose, D-xylose, and L-arabinose [1 , 2]. A 1% solution of GT has a viscosity of 3600 cps at 60 rpm using a Brookfield viscometer [1 , 2].

The potential of GT as an immobilization medium, has never been realized because at low concentrations, pertaining to a suitable void ratio, it suffers from viscosities far below levels necessary to support the mechanical stresses affected on a bead in reaction medium. Therefore an increase in the viscosity of GT gel is necessary before it can be used as an immobilization matrix. We have used ionomer formation, a mild procedure for increasing the molecular weight of polymers and therefore their viscosity, to this purpose. Further, we have selected a well-known reaction, the whole-cell penicillin G acylase system, as a model for evaluation of our new immobilization system. Penicillin G acylase is the key enzyme for the production of semi synthetic β-lactam antibiotics [5, 6, 7]. The penicillin acylase catalyzes the conversion of benzyl penicillin to phenylacetic acid PAA and 6-APA. Calcium alginate is one of the widely used natural polymers in immo-bilization of cells and enzymes but its mechanical stability is low and there are numerous reports regarding efforts to increase its mechanical stability [8]. *Chandy et al.* used the chitosan and polyacrylamid as a thin layer around alginate beads to increase its mechanical stability [9]. *Bielecki* and *Bolech* immobilized *E. coli* using alginate, carageenan, polyvinilalcohol, and polyacrylamid gels to determine the best gel for immobilization of this microbial cell. They found that the cells immobilized in natural gums had the higher shelf-life and cells immobilized within alginate beads showed highest activity [2]. Therefore, in order to gain an evaluation of

the performance of GT polymers, we use the results obtained by *Bielecki* and *Bolech* on immobilization of the same strain that we used, in the alginate beads using the same reaction system, as a reference. The effects of important factors such as temperature, pH and concentration on hydrolysis rate have also been investigated to achieve the maximum conversion of substrate.

MATERIALS AND METHODS
Chemicals

Gum tragacanth was obtained from Sigma Chemical Corp. (St. Louis, MO). A strain of *Escherichia coli ATCC 11105* was purchased from DMSZ co. (Germany). Penicillin G (potassium salt) was kindly provided by Iran Antibiotic Sazi co. (Sari, Iran). All other chemicals were supplied either by Merck AG. (Germany) or Sigma Chemical Corp. (St. Louis, MO) and were of analytical grade.

Cell Growth

The strain *E. coli ATCC11105* was maintained on nutrient broth with 15-25% glycerol at -70 °C. For each sample, cells were grown on a slope of nutrient-agar, and transferred to pre-culture medium consisting of 2.5 % (w/v) yeast extract, with a pH of 7.0 at 25 °C for 24 hours while shaking at 220 rpm. The inoculum was prepared from 10 % (v/v) pre-culture to the main culture consisting of 0.5 g/l yeast extract, 1.0 g/l NH_4Cl, 0.1 g/l KH_2PO_4, 1.0 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, and 2.0 g/l phenyl acetic acid (PAA), pH 7.0 at 25 °C. The composition of the mineral culture was optimized previously [5 , 10]. Cells produce the maximum penicillin G acylase (PGA) after 48 hours under the above mentioned conditions were harvested by centrifugation (6000 g, 0-4 °C, 40 min) and kept in 100 mM phosphate buffer pH of 7.8 at 4 °C before immobilization.

Molecular Weight Increase through Ionomer Formation and Immobilization

GT solution with suitable concentration, approxi-mately between 1% (w/v) of tragacanth powder, was prepared in Tris-HCl buffer pH 6.5, with 0.17% methyl and 0.03% propyl p-hydroxybanzoate. Preservatives are necessary for most gum solutions, and the choice will

depend on the finished product and the formulation [1]. The viscosity of solution reaches maximum in 24 h at 25 ^{o}C. This maximum may be obtained in about 2 h at 50 ^{o}C, and in 8 h at 40 ^{o}C [1 , 2]. Therefore, for assurance about final viscosity of the solution, and to avoid any increase in solution viscosity after immobilization, it is better to use the solution after it reaches the maximum viscosity. This solution is stable for 2 weeks at 0-4 ^{o}C.

A 1% solution of GT in deionized water is a gel with insufficient viscosity and cohesiveness to serve as an immobilization matrix. Several methods are available for increasing the molecular weight [11]. However, as this particular application involves live cells, the method used must require only the mildest of chemical and physical conditions if cells are to survive. Formation of ionomers offers mild reaction conditions for increasing the molecular weight. Ionomers are long chain of polymers that are cross linked by ionic bonds, rather than covalent bonds. The ionic bond is usually between an organic acid anion attached to the polymer and a metal cation. As a carbohydrate, GT contains carboxylic acid groups that can be used as anion cites with divalant metal cations, therefore for increasing the degree of cross-link in GT, Aluminum and Iron (III) were chosen as the cations; as they are non-toxic in concentrations used and their trivalent oxidation state helps increase the molecular weight three fold as each cation forms ionic bonds with GT chains [11].

For preparation of the immobilization mixture, 1 ml cell suspension and 1 ml of 10% (w/v) aqueous ferric chloride solution were added to 3 ml GT solution and mixed thoroughly to obtain a uniform mixture of solution. This mixture was dropped using a syringe into a 1% (w/v) aluminum chloride, which is slowly stirred, to form tragacanth beads spontaneously. Beads containing the immobilized cells were kept in $AlCl_3$ solution for 1 hour to reach the maximum stability. The beads were first washed with Tris-HCl buffer pH 6.5 and then with 0.9 % NaCl to remove the non-immobilized cells. The beads were kept in 4 ^{o}C until use. These beads were used for hydrolysis of penicillin G (potassium salt) substrate.

Penicillin G Hydrolysis

The penicillin acylase catalyzes the conversion of benzyl penicillin to PAA and 6-APA. Assay methods have been developed based on either of these products.

Batch hydrolysis of penicillin G was carried out in a stirred reactor showed in Fig. 2. The agitation speed for all experiments was 170 rpm, which was found to be the optimum agitation speed in previous investigations [5, 10]. Penicillin G was hydrolyzed to 6-APA, the key intermediate substance for semi-synthetic antibiotics such as amoxycillin and ampicillin, and PAA [7, 12, 13]. Each batch contained a certain number of beads containing immobilized *E. coli* cells and 50 ml of potassium salt of penicillin G in 100 mM Tris-HCl buffer as substrate.

Product concentration was measured using pH-stat and p-dimethylaminobenzaldehyde methods [11 , 14]. In the first method, the amount of PAA produced by the reaction is determined by measuring the amount of base added to the solution to suppress the effect of the generation of PAA and therefore keep the pH constant. In the second method, p-dimethylaminobenzaldehyde forms a colored Schiff's base with free amino acid group of 6-APA.

Optimization of Reaction Conditions

Reaction conditions such as substrate concentration, pH, temperature, and bead concentration were optimized to gain the maximum activity and substrate conversion. Conversion is defined as the total moles of 6-APA produced in the reaction mixture divided by the total moles of initial penicillin G. One unit of PGA activity is defined as the amount of enzyme required for producing one micromole of 6-APA per minute.

In optimization of one factor, all other factors are kept constant and the reaction is carried out at varying levels of the factor being optimized.

RESULTS AND DISCUSSION
Cell Culture Stability

Production of PGA in the main culture reaches the maximum value after 24 hours with an increase in pH to 7.85. These cells are then harvested and suspended in phosphate buffer. As the drop in the production rate is less than 3% over the period of 10 days, we conclude that cells were stable under these conditions for at least 10 days.

Comparison of Two Analytical Methods

The pH - stat and P-dimethylaminobenzaldehyde methods were both employed for the determination of penicillin G conversion [11 , 14]. Fig. 2 compares the

results obtained by these methods. The two methods show similar results and differ by 10 % in average based on the conversion. Since in the pH-stat method, pH of the reaction mixture is maintained at a constant value, and therefore it is in the favor of both the enzyme activity and stability, we chose the pH-stat method for the determination of reaction progress rate. As shown in this figure, the conversion of penicillin G increases with time and reaches a plateau after 6 hours of reaction.

Effect of pH

The decrease in pH of the reaction mixture during penicillin G hydrolysis is a result of deprotonization of PAA produced. This significantly reduces the reaction rate due to sensitivity of penicillin G acylase to a pH shift. Therefore, pH has to be carefully controlled to avoid the effect of reduced pH on the reaction rate. Based on a 6 h reaction time, the average hydrolysis conversion catalyzed by immobilized cells without pH control is more than 15% lower than that with the pH adjustment.

The effect of pH on the activity of the enzyme is measured by the time course of the substrate conversion at 40 $^{\circ}$C and pH values varying between 5.5 and 9. The conversion profile is shown in Fig. 3. The conversion of penicillin increases with an increase in pH and reaches 91.5% in 6 h at pH 6.5, however, further increase in pH results a drop in the conversion. The highest initial conversion rate is 40% in 1 h at pH 6.5.

The optimum operating pH for the soluble enzyme has been reported as 7.0 [10, 15]. The pH values reported for immobilized whole-cell PGA in polymeric matrix such as alginate, polyacrylamide, and so on, all exhibit a small shift from the pH of the soluble enzyme [16]. For example, for immobilization of the same strain within calcium alginate beads the optimum pH is reported as 7.5 [2], and for immobilization the same strain within polymethacrylamide beads the optimum pH value is reported to be 8.0 [16]. The reason for the apparent shift in the optimal pH might be due to interactions between the charged groups on the support material and hydrogen ions in the substrate solution.

Effect of Substrate Concentration

Hydrolysis of penicillin G at different substrate concentrations (1-8 %) was studied and the results are
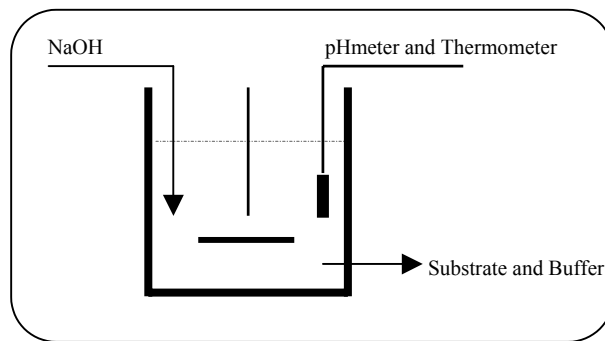

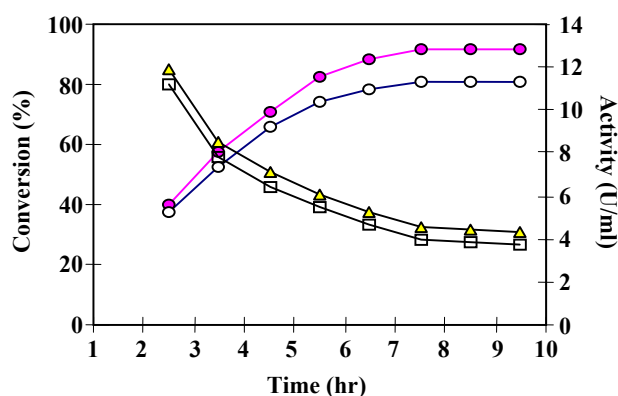*Fig. 1: Schematic diagram of the reactor used for hydrolysis of Pen G.*

Fig. 2: Comparison of two methods for determination of Penicillin G conversion. Conversion was determined by pH-stat method (●) and p-dimethylaminobenzaldehyde method (○); Activity of immobilized cells was also determined by pH-stat method (△) and p-dimethylaminobenzaldehyde method (□). Reactions were carried out at the optimum conditions (pH 6.5, 40 ^{0}C and 4% w/v substrate using150 GT beads containing immobilized cells).
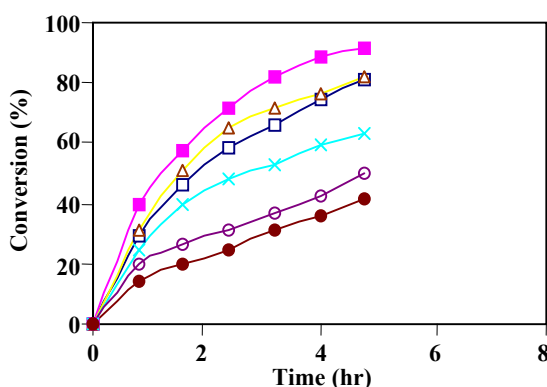

Fig. 3: Influence of pH on hydrolysis of penicillin G. (●) pH= 8.5; (○) pH= 8.0; (✗) pH= 7.5; (△) pH= 7.0; (■) pH= 6.5; (□) pH= 6.0. Reaction condition was 40 ^{0}C, 4% w/v substrate using 150 beads.
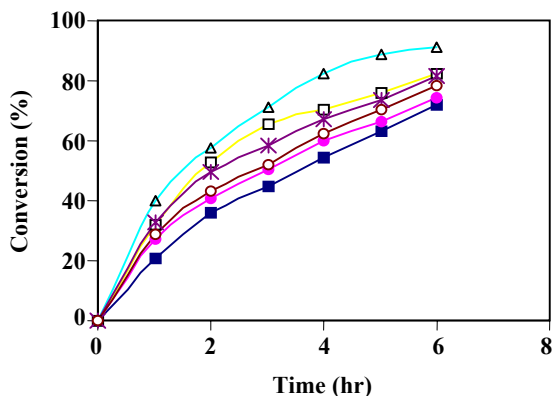
Fig. 4: Effect of substrate concentration (S) on conversion of penicillin G. (■) S = 1%; (•) S = 2%; (□) S = 3% (△) S = 4%; (✗) S = 6%; (○) S = 8%. Reactions were carried out at pH 7.5 and 45 ⁰C using 150 beads.

Fig. 5: Effect of the bead number on conversion of penicillin G. (□) 50 beads; (•) 100 beads; (■) 150 beads; (○) 200 beads; (△) 250 beads. Reaction condition was pH 6.5 and 40 ⁰C with 4% substrate.

shown in Fig. 4. The conversion of penicillin G increases with an increase in substrate concentration up to 4 %. However, a sudden drop is observed at substrate concentrations higher than 4 %. This phenomenon is due to the substrate inhibition effect, which has been verified by several workers for this enzyme [5,6, 14].

Our previous results obtained from alginate beads for immobilization of this strain show an optimum of 2 % substrate concentration for this system, whereas this value is 4% in the case of GT beads. These results indicate that GT beads can convert twice as much substrate in the same condition. This is due to the higher void volume of GT beads relative to alginate beads. The existence of galacturonic acid residues in GT causes high viscosities at low concentrations. For example, a 1% solution of unmodified high-grade GT has a viscosity of 3600 cps at 60 rpm using a Brookfield viscometer, while the same viscosity is achieved by a solution of 3% alginate. This leads to the advantage of higher porosity in GT beads, making it possible to immobilize a higher number of cells in a given volume, and reducing mass transfer limitations between the cells inside the beads and the medium.

Effect of Bead Concentration

The concentration of the immobilized beads is an important factor in hydrolysis of penicillin G. Fig. 5 shows time course of the conversion of penicillin G at different bead concentrations. The conversion reaches a maximum when 150 beads are suspended in 50 ml of
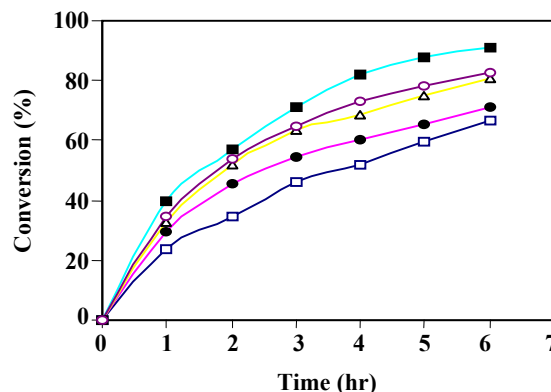
substrate solution. Further increases in concentration result in a drop in conversion. This can be due to the fact that both of products of this reaction have an inhibitory effect on the enzyme, as previously observed by other researchers [11, 14]. Therefore, the high rate of reaction at initial time when more than 150 beads are used, results in an initial buildup of both products and inhibits the enzyme so that a lower rate is observed throughout the reaction.

Effect of Temperature

The relationship between temperature and the activity of the immobilized whole-cell PGA was determined by measuring the reaction rate at optimum conditions and varying temperature 30 to 55 °C. Fig. 6 shows that the conversion increases with temperature up to 40 °C. However, increasing the temperature above 40 °C lowers the conversion. Even though, higher temperature favors the reaction kinetics and the diffusion of both substrate and products through the beads, it also results in denaturation of the biomaterials above a certain level. Using temperatures higher than 45 °C seems to have an inhibitive effect on the cells. This is likely to be due to the low stability of the enzyme and the very unstable nature of Pen G at high temperatures.

A slight shift to higher temperature in optimum operating temperature is usually observed between the free cells and the immobilized cells [6, 16]. It has been observed a +8°C shift for calcium alginate and a +3°C for GT. Since operation in higher temperature is
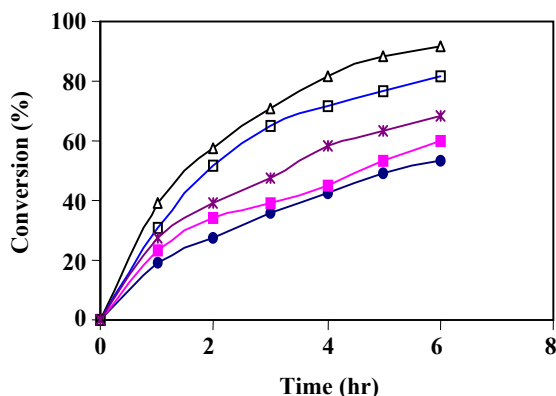
Fig. 6: Effect of temperature on conversion of penicillin G. (●) 30 ^{0}C; (■) 35 ^{0}C; (△) 40 ^{0}C; (□) 45 ^{0}C; (✗) 50 ^{0}C. Reactions were carried out at pH 6.5 and 4% w/v substrate using 150 beads.

Fig. 7: Effect of repeated use of immobilized cells (✗) within the GT beads and (+) within the Alginate beads, on conversions of penicillin G. Reactions were carried out at the optimum condition.

destructive to cells, the lower temperature shift observed in GT, is an advantage in stability of the enzyme and the cell. In addition, the particular substrate used in this reaction is also sensitive to temperature and therefore a lower temperature shift required for optimization of GT-immobilized cells, provides for a higher stability of the substrate.

Repeated Use of Immobilized Whole Cell

The operational stability of PGA immobilized in GT beads, which is an important issue in industrial application of immobilized cells or enzymes, was studied batch-wise in a stirred tank reactor with 4% Pen G solution. Each batch of experiments was performed at the optimum conditions for 6 h. The immobilized cells were then recovered, and kept in a solution of 1% AlCl$_3$ until the next incubation. As shown in Fig. 7, the cellsimmobilized using calcium alginate show a sharp drop in activity after the 25th run. This is due to the low mechanical stability of alginate beads resulting in disintegration of beads after 25 runs. However, the same cells immobilized using GT retain greater than 85% of their initial activity after 50 runs. The experiments were stopped after 50 runs.

CONCLUSIONS

A new medium for immobilization of live cells for catalysis of bioreactions is introduced. A 1% solution of Gum Tragacanth in water, which is usually a gel, is thickened by increasing the molecular weight through
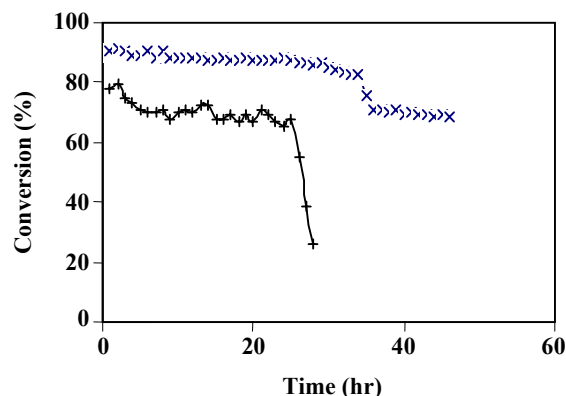
ionomer formation with trivalent cations. This medium, besides offering biocompatibility, pH stability, and ease of use, properties usually sought in mediums for immobilization, offers a very high void ratio, and significantly higher mass transfer coefficients which leads to high overall conversion rates. Also, GT exhibits better long term mechanical stability under reaction conditions.

To evaluate the performance of GT, we used a well-known reaction (the conversion of benzyl penicillin to 6-aminopenicillanic acid by means of *Escherichia coli ATCC11105*) and immobilized the same strain of *E. coli* both in GT and calcium alginate (a well-known matrix). Comparison of results shows that not only GT serves well as a matrix for immobilization, but also shows enhanced activity and stability. This indicates a strong potential for an increase in performance of other enzyme catalyzed reaction systems if GT is used as the immobilization matrix.

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