COMPARISON OF IMMOBILIZED WHOLE RESTING CELLS IN DIFFERENT MATRICES VIS-A-VIS FREE CELLS OF *Bacillus megaterium* FOR ACYLTRANSFERASE ACTIVITY

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ABSTRACT

The bacterial isolate *Bacillus megaterium* was isolated from the soils of contaminated site of pharmaceutical industry, Sitapura, Jaipur and identified as an amide hydrolysing bacterium. Amidase, an amide-hydrolyzing enzyme also shows acyltransferase activity. The use of acyltransferase or bacteria with microbial enzymes with acyltransferase activity may be used to convert amides to Hydroxamic acids. The whole resting cells of *Bacillus megaterium* were prepared and assayed for acyltransferase activity spectrophotometrically. Known amount of whole resting cells of *Bacillus megaterium* were immobilized in the gel beads of sodium alginate, agar, polyacrylamide and PVA-alginate. These immobilized beads were used repeatedly as biocatalysts in 10 reactions to test their reusability potential. The whole cells immobilized into four different matrices were tested for the thermal stability and reusability potential in comparison to free cells. This study proved useful in understanding the technique of immobilization of acyltransferase enzyme, its operational stability and its importance in the synthesis of hydroxamic acid.

Key Words : Immobilized cells, Acetamide, Hydroxamic acid, Acyltransferase, Alginate, Agar polyacrylamide, PVA-alginate

INTRODUCTION

Amidases are ubiquitous enzymes and biological functions of these enzymes vary widely. With the help of amidase acyltransferase activity, microbes synthesise hydroxamic acids and exploit their chelating properties to obtain iron (which is an important component of cytochromes and iron sulphur proteins) from iron deficient environments. Amidases also have applications in the biodegradation of toxic amides and nitriles from polluted sites[1]. These compounds are often released directly to the environment after passing through wastewater treatment processes (via wastewater treatment plants or domestic septic systems) which often are not designed to remove them. Hence, for the safety of the human environment, this study focuses on microbial degradation of dyes and drugs which are amide compounds[2] and this includes finding microorganisms having amide degrading ability viz amidase activity[3]. Immobilization was tried with whole cells containing acyltransferase enzyme on four different matrices such as agar gel, sodium alginate, polyacrylamide gel and PVA. Immobilized cells were then studied for their reusability and thermostability potentials. Gilian *et al*(1993)[4] reported that amidase from *R. equi* TG 328 was stable upto 8 h at 50^0C but the amidase from *Rhodococcus rhodochrous* J1 was incubated for 30 min in 10 mM phosphate buffer (pH 7.5) and it was found that this enzyme was completely stable upto 30°C but was unstable at 55^0C[5]. Acyltransferase activity of amidase has been used for the biosynthesis of a wide range of hydroxamic acids which have a high chelating potential. In acyltransferase catalyzed biotransformation, amides acts as acyl-group donors and

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hydroxylamine as acyl-group acceptors **(Fig. 1)**. Several hydroxamic acids are used as drugs since they are constituents of growth factor, antibiotics, antagonists, tumor inhibitor, antifungal agents, and cell dividing factors; indeed they are known to inhibit enzymes such as metalloproteases which are commonly involved in animal and human disease and development[6]. In most of the industrial, analytical and clinical processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the exhaustion of the substrates. This single use is obviously quite wasteful when the cost of enzyme is considered. Thus, there is an incentive to use enzyme or cells in an immobilized or insolubilized form so that they may be retained in a biochemical reactor to catalyze further the subsequently added substrate. The use of an immobilized enzyme or cells makes them economically feasible to operate an enzymatic process in a continuous mode.

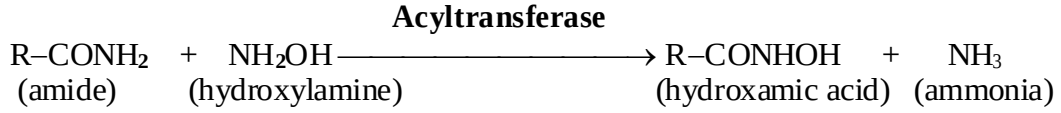

Fig.1 : Acyltransferase cataltzed biotransformation

MATERIALS & METHODS

Chemicals

Acetamide, hydroxylamine and Acetohydroxamic acid were obtained from Hi Media Chemicals Ltd, India. Peptone and yeast extract were purchased from Suyog Diagnostics Private Ltd (New Delhi, India). All other chemicals used were of analytical reagent grade purity and were obtained from Merck, India.

Isolation of microorganisms from soil by use of acetamide enriched medium

The soil samples collected from effluent sites of pharmaceutical industries located in Sitapura area respectively in Jaipur, Rajasthan India were pooled and transported in sterile containers and stored at 4°C. Bacteria was isolated from soil sample[7] earlier by an enrichment culture procedure and identified as Bacillus megaterium F-8 through biochemical tests[8-9] and phylogenetic studies[10]. Enrichments were carried out in the Nitrogen-free minimal medium containing 2 g of acetamide as nitrogen source. The Nitrogen free minimal medium contained (g/l) : Acetamide, 2, Glycerol,4 KH_2PO_4,1; K_2HPO_4,1; NaCl,1; $MgSO_4.7H_2O$,0.2; $FeSO_4.7H_2O$,0.01; pH7.2.

Preparation of resting cells

Bacillus was inoculated into 50 ml of modified nutrient broth containing 5 g peptone, 1 g yeast extract, 1 g beef extract, and 10 g glucose per litre of distilled water pH 7.2 and incubated at 30°C for 24h in an incubator shaker at 175 rpm[11]. These 24 h preculture were added to 50 mL of production medium containing 30 g tryptone, 15 g yeast extract, 5 g NaCl per litre of water, pH 7.5 followed by incubation at 30^0C for 24 h in an incubator shaker at 175 rpm. Then culture was centrifuged at 5000 g for 15 min at 0-4^0C. The cell pellets were suspended in 0.1M solution phosphate buffer (pH 7.0) after two washing with the same buffer. These cell suspensions were referred to as 'resting cells' having concentration of 300 mg/mL (wet weight). They were assayed for amidase and acyltransferase activity and used for further investigations[12].

General assay conditions and determination of enzyme activity

The colorimetric screening method was based on amidase catalyzed synthesis of hydroxamic acid. When an acidic ferric chloride solution was added to the reaction mixture, color changed from light yellow to magenta which indicated the occurrence of a positive reaction. The colorimetric screening method was performed in a similar manner to the assay of acyl transfer activity established[13]. The reaction medium consisted of an aqueous solution of acetamide adjusted to pH 7 with 10M NaOH (2m*l*; 400mM), hydroxyl amine hydrochloride, adjusted to pH 7 with 10M NaOH (2mL; 2M); sodium phosphate buffer pH 7 (2mL; 100mM); and resting cells (2mL). The reaction was performed at

30°C. One unit of acyl transfer activity was defined as the quantity of enzyme required for production of 1µmol/min of acetohydroxamic acid at 30°C and pH 7.0.

Acetohydroxamic acid was assayed using the method developed based on the colorimetric determination of the resulting red brown complexes with Fe (III).

Samples of 0.5 ml reaction medium were taken at regular intervals (every 4 min) and immediately assayed by adding 1ml of iron chloride solution (21 ml $FeCl_3$ 27.5% (W/V), 5.3 ml (12.5M) HCl, to 100 mL distilled water). This stopped the reaction and yielded a stained complex. Absorbance was measured at 500 nm. The molar absorptivity of the iron (III)/2,2 dimethylcyclopropane hydroxamic acid complex was 3.34 x 102 $mol^{-1}cm^{-1}$. Reaction mixture without resting cells was also tested to exclude any possible spontaneous chemical synthesis of hydroxamic acid.

Immobilization of cells of Bacillus:

According to Kierstan and Bucke (1977)[14] 10 ml of resting cells (200 mg/ml) were mixed thoroughly with sodium alginate solution (15 ml, 3.3 W/V). The resultant suspension was dropped into a magnetically stirred solution of $CaCl_2$ (0.2M) to obtain spherical beads. The beads were stirred further for 1 h, washed and stored at 4^0C. In case of immobilization in agar, 1ml resting cells (200 mg/ml) were added to 9 mL sodium phosphate buffer (100 mM, pH 7) and thoroughly mixed with 2.5% of 100 ml agar solution. The mixture was immediately poured into a petridish and kept at 50°C for solidifying. The gel was cut into beads of 1 cm diameter and stored in sodium phosphate buffer at 4°C for further use[15]. For immobilization in polyacrylamide to 8 ml of sodium phosphate buffer (pH 7.0), 1.5 g of acrylamide, 0.75 g of bis-acrylamide and 5 mg of ammonium persulphate were added thoroughly mixed and heated. 50 µl of TEMED was added to this test tube. In another test tube, 2 ml of resting cell suspension (200 mg/ml) was thoroughly mixed with 3 m*l* chilled sodium phosphate buffer. The contents of the two test tubes were mixed properly and immediately poured in petridish and covered. Polymerization was allowed to proceed for 1 h. The solidified gel was cut into beads of 1cm diameter and stored in the same buffer at 4^0C for further use[16]. For immobilization in polyvinyl alcohol-alginate[17], 20% (W/V) PVA and 15% (W/V) alginate were mixed in 20 mL of distilled water and heated to 60^0C to completely dissolve the PVA[11]. The solution was then cooled to 35^0C and thoroughly mixed with 5 ml resting cell suspension (200 mg/ml). The resultant suspension was dropped into a magnetically stirred solution of $CaCl_2$ (0.2M) and immersed for about 2 h to form spherical PVA-alginate beads.

Assay of acyl transferase activity of immobilized cells

According to Brammer and Clarke[17-18] and Fournand *et al.,* immobilized cells were incubated with amide and hydroxyl amine and sodium phosphate buffer at 37^0C for 1 hr. After the incubation the beads were removed by filtration. The filtrate was centrifuged at 10000 g for 30 min. to remove the fine particles. The clear supernatant was then assayed for acyl transferase activity.

Reusability of Alginate, Agar, Polyacrylamide and PVA alginate immobilized cells for acyl transferase activity was determined

The immobilized beads were used repeatedly as biocatalysts in 10 reactions to test their reusability potential. Numbers of beads containing an equivalent enzyme as present in given resting cell suspension were added in each reaction mixture and removing the beads from the reaction mixture (instead of using $FeCl_3$ reagent) stopped the reaction. The thermostability of the most suitable matrix was also studied.

Optimization of reaction conditions

Reaction conditions such as pH, temperature, substrate concentration and type of buffer for free resting cells and immobilized cells in most suitable matrix were optimized[20].

RESULTS AND DISCUSSION

Selection of a suitable matrix for acyltransferase of immobilized resting cells of *Bacillus megaterium* F-8

Resting cells of *Bacillus megaterium* F-8 were efficiently immobilized by entrapment in agar, polyacrylamide, alginate and PVA-alginate gel

(Fig 2 to Fig 5) and assessed for their reusability potentials. Initially, entrapped resting cells exhibited about 50%, 63% and 58% residual enzyme activity in agar, polyacrylamide and PVA alginate gels respectively, however it was increased to about 72%, 50% and 82% after fifth recycle. Alginate gel beads containing resting cells of isolated bacterium were recycled for ten cycles and it was observed that there was no loss of activity. PVA alginate and alginate gel discs showed better reusability in comparison to agar and acrylamide gel beads while alginate gel beads showed maximum reusability **(Fig 6)**.

Optimization of reaction conditions for assay of acyl transferase activity of alginate immobilized bacterial cells

There was no significant change in type of buffer, pH, temperature and substrate concentration of alginate immobilized cells w.r.t. free cells. Acyltransferase activity of alginate gel entrapped resting cells of *Bacillus megaterium* F-8 was assayed at a pH range from 5.0 to 10.0.

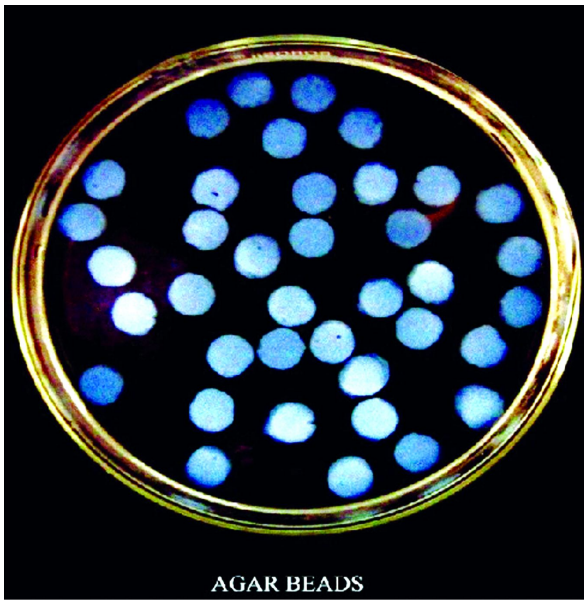

Fig 2 : Plate showing resting cells in agar gel discs

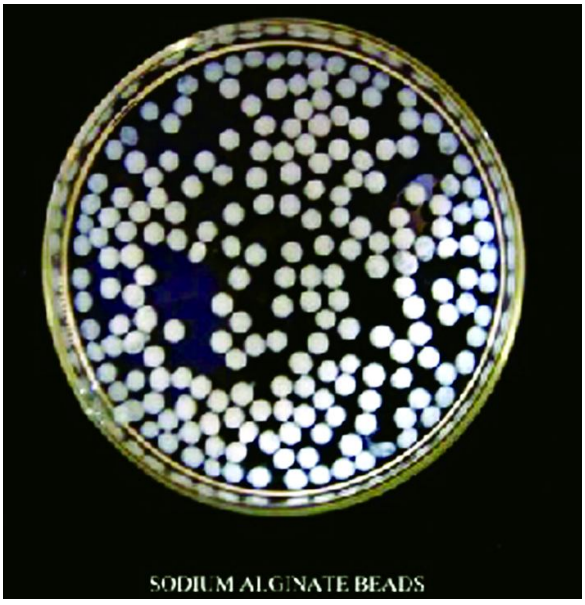

Fig 3 : Plate showing resting cells of alginate gel beads

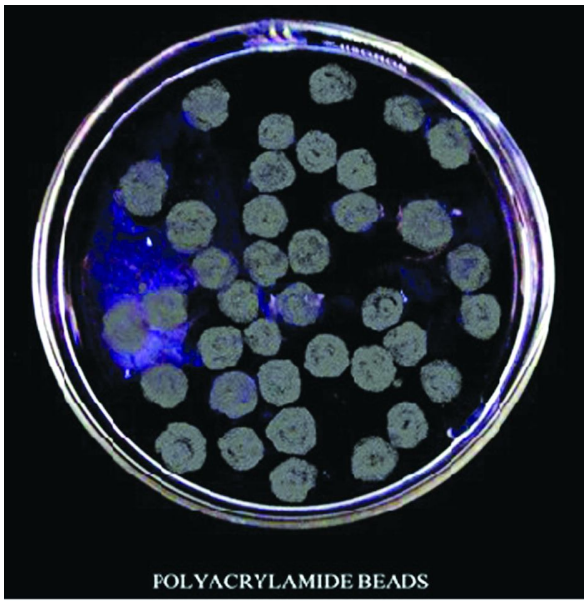

Fig 4 : Plate showing resting cells in polyacrylamide gel discs

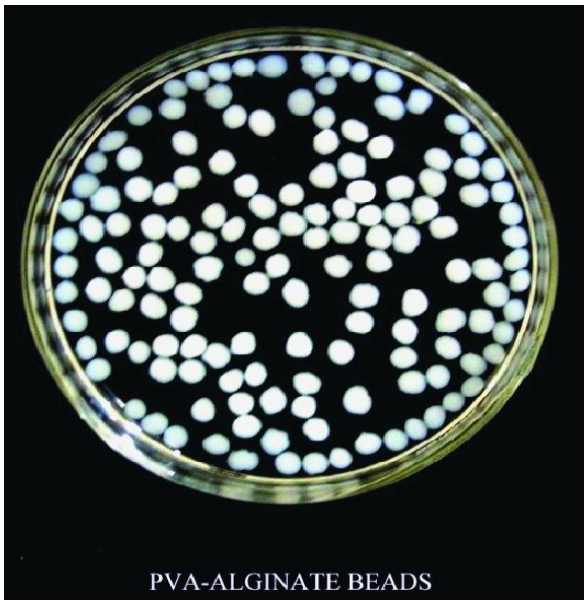

Fig 5 : Plate showing resting cells of PVA- alginate gel beads

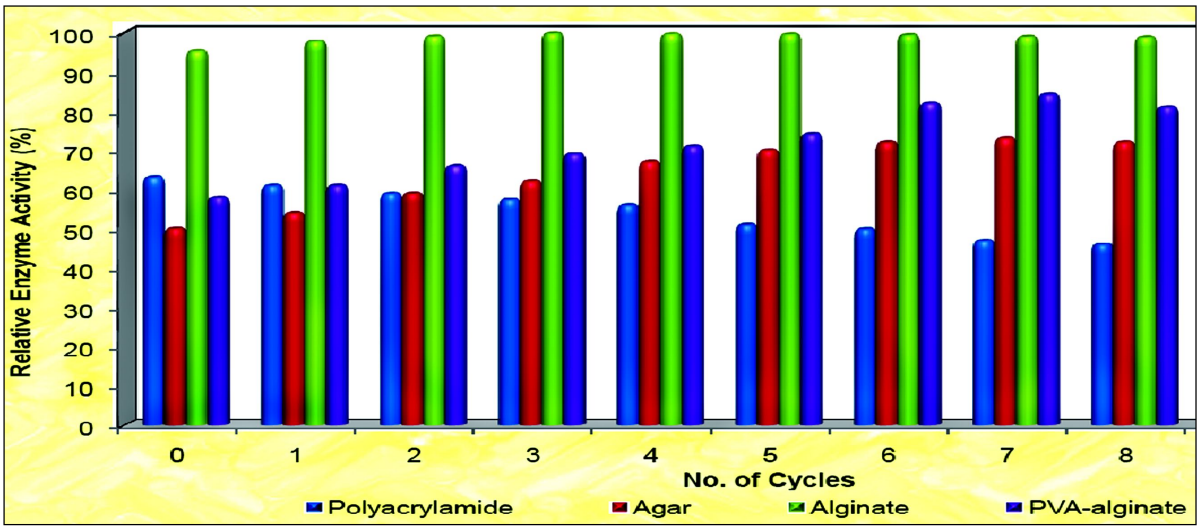

Fig 6 : Comparison of relative enzyme activity of immobilized beads on 4 different matrices for 10 recycles

Maximum acyltransferase activity was found at pH 7.5 while free cells exhibited 8.0 as pH optima for amide hydrolysis. Among the four buffers tested, Sodium Phosphate buffer gave the best results with acyltransferase of immobilized *Bacillus megaterium* F-8 cells. Comparable activity was observed in potassium phosphate and glycine-NaOH buffer except Tris-HCl buffer where 50% of relative enzyme activity was recorded. Sodium phosphate buffer showed maximum enzyme activity with immobilized cells while potassium phosphate proved to be slight better to sodium phosphate buffer with free cells. Optimum substrate concentration for alginate gel entrapped cells was 1.0 mmoles which is higher compared to the optimum substrate concentration of 0.850 mmoles for free cells. However, at further higher concentration of the substrate, enzyme activity remained more or less constant. Maximum acyltransferase activity was exhibited at 55°C by both alginate immobilized and non-immobilized *Bacillus megaterium* F-8 cells. Relative enzyme activity (%) recorded at different temperature was also more or less identical in both immobilized and free cells.

Thermostability of alginate gel beads was investigated by preincubating them at different temperatures in the range of 35°C to 85°C for 120 minutes. The entrapped resting cells retained very good activity up to 55°C. In comparison to free cells, alginate gel immobilized cells exhibited higher thermostability probably due to inert nature of alginate and also endothermic gelling reaction. Biocatalyst thermostability is of paramount importance for any bioprocess[12].

CONCLUSION

The results obtained in this investigation show that *Bacillus megaterium* F-8 entrapped in alginate gel discs express thermostable acyl transferase activity that may be used repeatedly for the hydrolysis of amides and synthesis of hydroxamic acids. Biotransformation at commercial scale has a very high potential in contrast to chemical processes for the synthesis of this commodity compound which is a key compound in medicine, analytical chemistry, phytochemistry, agronomy and in nuclear technology and waste water treatment studies.

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