

Original Research Article

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Immobilized Polyextremophilic α -Amylase of *Bacillus mycoides* for Citric Acid Production Using Starch

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ABSTRACT

Immobilization is a wide spread technique in the last three decades. It is a powerful method which can improve properties of enzymes like stability, activity, resistance to inhibition due to the by-products in fermentation and its quick recovery. In the present investigation a halophilic organism *Bacillus mycoides* producing α -amylase was isolated from saline water. A mixed fermenter system was made for citric acid production using starch. α -amylase of *Bacillus mycoides* was used for conversion of starch into glucose and further *Aspergillus niger* was used for conversion of glucose into citric acid. The enzyme activity of free enzyme and free cells was assayed by DNSA method. Effect of gel concentration, cell concentration and enzyme concentration was studied for both immobilized cells and enzyme. The activity of immobilized enzyme was high by 78% as compared to that of immobilized cells at 1.5% of gel concentration. Activity of enzyme and cell was high at 1.5ml of its concentration. The activity of enzyme was high as compared to cell by 75.5%. It was found that the enzyme and cells were stable and gave high activity at 6.0pH and 60°C after immobilization. Then under all optimum conditions immobilized beads of *Bacillus mycoides* cells and α -amylase beads were paced in 20% starch solution and beads of *Aspergillus niger* were added into it for citric acid production. The productivity for starch hydrolysis and citric acid was then checked in different bioreactors and was found maximum in Air lift- fermenter which was 73% and 80% high as compared with shake flask and column reactor.

Keywords

Immobilization, α -amylase, Air lift reactor, Column reactor, Citric acid

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Introduction

The term immobilized enzymes refers to enzyme physically restricted or constrained in a certain defined region of space with retention of their catalytic activities and which can be used sustainably. The constituents of an immobilized enzyme system consist of the enzyme, the matrix and the mode of attachment. The attachment of the enzyme to

the support depends upon the stability of the covalent bonds by reversible physical adsorption and ionic linkages (Brena Breatriz *et al.*, 2006).

Immobilized enzyme was discovered since 1916, when Nelson and Griffin discovered that invertase when adsorbed to charcoal has the ability to hydrolyze sucrose (Ahmad Razi *et al.*, 2015).

The reusability and stability of the immobilized enzyme was identified by Grubhofer and Schelth, reported the covalent immobilization of various enzymes (Nisha *et al.*, 2012). The easy contact with the substrate and non-detachable from the inert matrix support, increases the benefits of immobilization. It is a technique which is preferred over free enzyme catalysis giving the advantages like increasing enzyme's stability, easy separation of reactant and product, repeated use of a single batch of enzyme which eventually saves the enzyme, toil and overhead costs (Maheshwari Uma *et al.*, 2014).

Adverse conditions are too harsh for normal life to exist, but a variety of bacteria and fungi can survive.

These organisms have evolved to exist in these extreme environment and fall into a number of different categories, including halotolerant, moderate, borderline and extremely halophilic (Esawy Mona Abdeltawab *et al.*, 2014). Halophilic bacteria are commonly found in natural environments containing significant concentration of NaCl (Irshad Aarzo *et al.*, 2013).

Among halophilic microorganisms are a variety of heterotrophic and methanogenic archaea; photosynthetic lithotrophs, and heterotrophic bacteria and photosynthetic and heterotrophic eukaryotes (Azhar Mohsin *et al.*, 2014). Comparatively halophilic organisms grow optimally between 0.5-2.5 M salt concentration (Todkar Sandip *et al.*, 2012). The word halophile is derived from Greek meaning "salt loving" (Kumar Sumit *et al.*, 2012).

Amylases are normally constitutive enzymes. Very few alpha amylases have been studied from halophilic origin (Kumar Sumit *et al.*, 2012). Amylases are enzymes, which

hydrolyze starch molecule to give diverse products including dextrin and progressively smaller polymers composed of glucose units (Singh Pushpendra *et al.*, 2012). Amylases are widely present in microorganisms, plants and animals, and have found applications in numerous industries including starch liquefaction, brewing, food, paper, textile and pharmaceuticals (Tavano Olga Lusia *et al.*, 2013; Abdu Al- ZaZae Mohammad *et al.*, 2011).

Two major classes of amylases have been identified namely α -amylase and β -amylase (Prabhakaran *et al.*, 2009). α -amylase is most abundantly found in humans and other mammals (Raghu *et al.*, 2015). The production and thermo-stability of α -amylase is highly dependent upon the type of strain, composition of media, method of cultivation, cell growth, nutrient requirement, metal ions, pH, incubation temperature and time of incubation. The enzyme has been shown to be activated in presence of Ca^{2+} and K^{+} while inhibited by Co^{2+} and Cu^{2+} (Abdu Al- ZaZae Mohammad *et al.*, 2011).

In this study, the efforts have been made to isolate the halophile with good α -amylase activity in the presence of starch as a carbon source.

The parameters influencing the amylase productivity such as time, temperature and pH were studied. The enzyme was partially purified and successfully immobilized in sodium alginate. The parameters influencing the amylase activity of immobilized enzyme such as sodium alginate concentrations, enzyme concentrations and cell concentrations were studied. The unconventional production of citric acid from starch using immobilized bacterial isolate and immobilized *Aspergillus niger* in different bioreactors such as shake flask, column reactor and air-lift reactor was studied.

Materials and Methods

Isolation and screening of bacteria

The sea water sample collected from sea shore was enriched in selective medium. For primary screening, isolates were streaked over the starch medium containing 1% starch, 0.5% yeast extract, 0.5% peptone, 0.01% MgSO₄, 5% NaCl and 2% agar at pH 6.0 for the selection of starch degrading organism. The plates were incubated at 30°C for 24 hrs and were flooded with Gram's iodine solution (2% iodine and 0.2% potassium iodide) (Abdu Al-ZaZae Mohammad *et al.*, 2011; Suman *et al.*, 2010). Organism giving largest hydrolysis zone was considered.

Characterization of isolated organism

The characterization of isolated organism was done by its morphological and biochemical characters, Gram's staining and identified as per Bergey's manual and further conformed by VITEK 2 system version 05.02. The *Aspergillus niger* NCIM-1246 was procured from NCL, Pune, India.

Polyextremophylic nature of organism

The polyextremophylic nature of isolate was determined by culturing in nutrient medium with varying salt concentrations (0.5%, 2%, 5% and 10%), at different temperature (30, 40, 50, 60 and 70°C) and pH (5, 6, 7 and 8).

Fermentation process

For large scale production of the alpha amylase, fermentation was carried out in an baffled Erlenmeyer flask containing starch medium of composition - 20% starch, 0.5% yeast extract, 0.5% peptone, 0.01% MgSO₄ and 5% NaCl adjusted at pH 6.0. The flasks were sterilized and then seeded with 5% inoculum of 24hrs old bacterial culture and

incubated at 30°C for 72hrs (Mostafa Yasser *et al.*, 2014) on shaking incubator.

Partial purification of enzyme

The fermented broth was centrifuged at 5000rpm for 20mins at 4°C. The supernatant (crude enzyme) was further used for salt precipitation by 80% ammonium sulphate. The precipitate was centrifuged at 10,000rpm for 20mins at 4°C. The resultant pellet was then dissolved in 0.2M PO₄ buffer (pH8), dialyzed and lyophilized (Abdu Al-ZaZae Mohammad *et al.*, 2011).

α -Amylase enzyme assay

α -Amylase was assayed as described by kirankanthi *et al.*, 2012. The activity of α -amylase was determined by using starch as the substrate. The amount of reducing sugar released was measured by using 3, 5-dinitrosalicylic acid using maltose as the standard (Lahiri Preeti *et al.*, 2015; Kiran Kanthi *et al.*, 2012). In this study, 0.5ml of starch solution (20% w/v) in phosphate buffer and 0.5ml of enzyme solution was incubated at 30°C for 10mins in hot water bath. The enzymatic reaction was stopped by adding 1ml of DNSA reagent and incubated in boiling water bath for 15mins and absorbance was taken at 530nm. An enzyme blank with DNSA added prior to enzyme addition served as control. Effect of enzyme activity at different pH (3-8) and temperature (30-70°C) was studied.

Immobilization of halophile

The alginate entrapment of cells and enzyme was performed according to the method suggested by Mustafa Yasser *et al.*, (2014). One ml of cell suspension (4×10^8 CFU/ml gel) was mixed in 2% alginate solution. Beads of this solution were made in 3.5% (w/v) CaCl₂ solution on a magnetic stirrer. In this study,

the parameters influencing the activity of α -amylase enzyme such as sodium alginate gel concentrations (1.5%, 2% and 2.5%), cell concentrations (2×10^8 CFU/ml, 4×10^8 CFU/ml, 8×10^8 CFU/ml), and enzyme (partially purified enzyme) concentrations (0.4ml, 0.6ml and 0.8ml) were studied.

Immobilization of *Aspergillus niger*

Aspergillus niger was grown in Sabouraud Dextrose Broth at 28°C for 48hrs and then medium was centrifuged at 5000rpm for 15mins at 4°C. The pellet was suspended in Tris buffer. One ml of this suspension (3×10^9 spores) was mixed in 2% alginate solution and beads were made in 3.5% CaCl₂ solution.

Design and operation of bioreactors

Shake flask

Shake flask also called as Erlenmeyer flask. 1% starch as substrate was added into flask along with immobilized beads of halophilic organism, kept on shaker at 200rpm.

Sample was withdrawn after every 24hrs and checked for formation of glucose by DNSA method.

Immobilized *Aspergillus niger* are then added to the flask and then incubated on shaker and production of citric acid was assayed gravimetrically following Marrier and Boulet method (Pandey *et al.*, 2013).

Column reactor

Column reactor consists of a column of 10inch in length and 0.5 inch width. The column was filled with immobilized beads. Substrate was than filled in the column and kept in contact with the beads without shaking. Sample was withdrawn after every 24hrs and checked for substrate conversion.

Airlift reactor

It is also called as bubble column reactor. It has column, immobilized bed and has an additional apparatus called sparger for aeration. Sparger was placed at the bottom of the column, it was than filled with immobilized beads. Column was than filled with substrate *i.e.*, 20% starch solution and continuously supplied with air. Samples were withdrawn after every 24hrs and checked for glucose production.

Results and Discussion

Isolation and screening of the bacteria

Four isolates (A₁, A₂, A₃ and A₄) of halophilic bacteria were obtained. During the screening the isolate A₄ gave the maximum hydrolysis zone of 17mm and this organism was then further characterized.

Characterization of isolated organism

The isolated organism was identified as *Bacillus mycoides* and it was the same species as reported by Suribabu *et al.*, (2014). The identification of organism by VITEK system is shown in table 1.

Polyextremophylic nature of organism

The organism was found to be thermophilic tolerating 60°C temperature and grow well at pH6.0 with 5% of salt concentration indicated its polyextremophilic nature.

Enzyme activity of free α -amylase and free cell

The enzyme activity of free enzyme was high as compared to free cell. The enzyme activity was high after the incubation of 48hrs and further the decrease in enzyme activity was observed (Table 2 and Fig. 1).

Table.1 Identification of potent isolate by VITEK system

Selected Organism	91 % Probability	<i>Bacillus mycoides</i>				
Organism	Bionumber: 4337001150642621					
identification		Confidence: very good				
Contraindicating Typical Biopattern (s)						
<i>Bacillus mycoides</i> BNAG(92), LeuA(79), APPA(17), BMAN(22).						
Biochemical details:						
1	BXYL - 3	LysA - 4	AspA (+) 5	LeuA + 7	PheA + 8	ProA -
9	BGAL + 10	PyrA + 11	AGAL - 12	AlaA + 13	TyrA + 14	BNAG +
15	APPA - 18	CDE X - 19	dGAL - 21	GLYG - 22	INO - 24	MdG (-)
25	ELLM + 26	MdX - 27	AMAN - 29	MTE + 30	GlyA - 31	dMAN -
32	dMNE + 34	dMLZ - 36	NAG + 37	PLE - 39	IRHA - 41	BGLU -
43	BMAN - 44	PHC + 45	PVATE (+) 46	AGLU (-) 47	dTAG - 48	dTRE +
50	INU (-) 53	dGLU + 54	dRIB - 55	PSCNa - 58	NaCl 6.5% + 59	KAN +
60	OLD - 61	ESC + 62	TTZ - 63	POLYB_R +		
Installed VITEK 2 System Version: 05:02						

Table.2 Enzyme activity of free α -amylase and free cell

Time in hrs	Enzyme activity (mM/ml/hr)	
	Free enzyme	Free cell
0 hrs	00.30	00.12
24hrs	21.42	19.08
48hrs	25.30	22.56
72hrs	13.08	11.48

Table.3 Effect of gel concentration on enzyme activity (mM/ml/hr).

Gel conc. / Time in hrs	1.5%		2%		2.5%	
	Immobilized cells	Immobilized enzyme	Immobilized cells	Immobilized enzyme	Immobilized cells	Immobilized enzyme
0hrs	00.02 ± 0.02	00.02±0.03	00.01 ± 0.03	00.04±0.01	00.03 ± 0.01	00.01±0.02
24hrs	14.50 ± 0.08	18.23±0.06	11.42 ± 0.05	16.37±0.02	05.58 ± 0.06	11.83±0.04
48hrs	16.37 ± 0.05	20.22±0.04	15.33 ± 0.07	19.51±0.08	10.37 ± 0.04	15.92±0.08
72hrs	05.33 ± 0.03	10.13±0.08	04.08 ± 0.03	8.073±0.05	02.14 ± 0.08	7.739±0.02

Table.4 Effect of cell concentration and enzyme concentration on enzyme activity (mM/ml/hr).

Concentration Time	0.5ml		1ml		1.5ml	
	Cell	Enzyme	Cell	Enzyme	Cell	Enzyme
0hrs	00.2±0.02	00.02±0.01	00.04±0.03	00.01±0.02	00.06±0.02	00.02±0.01
24hrs	07.91±0.05	11.67±0.03	09.58±0.01	15.41±0.05	13.75±0.01	17.5±0.02
48hrs	10.49±0.03	13.54±0.02	12.67±0.03	16.45±0.04	15.41±0.08	20.41±0.06
72hrs	04.58±0.01	05.13±0.05	05.83±0.08	06.38±0.05	07.08±0.03	07.78±0.01

Table.5 Productivity of immobilized enzyme and immobilized cells in different bioreactors

Time in hrs	FC	FE	Shake flask		Column reactor		Airlift reactor	
			IC	IE	IC	IE	IC	IE
0hrs	00.12	00.30	00.02 ± 0.02	00.02±0.03	00.03±0.01	0.01±0.02	0.03±0.01	0.02±0.01
24hrs	19.08	21.42	14.50 ± 0.08	18.23±0.06	07.48±0.03	14.27±0.01	17.39±0.05	23.65±0.04
48hrs	22.56	25.30	16.37 ± 0.05	20.22±0.04	10.76±0.02	16.97±0.5	22.82±0.08	27.61±0.07
72hrs	11.48	13.08	05.33 ± 0.03	10.13±0.08	08.15±0.01	11.03±0.04	12.71±0.03	17.38±0.02
Citric acid (mg/ml) After 8 days of fermentation	102	114	66	78	42	58.5	85.5	91.5

Abbreviations: IC- Immobilized cells, IE- Immobilized enzyme, FC- Free cell, FE- Free enzyme

Fig.1 Effect of gel concentration using Immobilized cells on enzyme activity. **Fig.2** Effect of gel concentration using immobilized enzyme on enzyme activity.

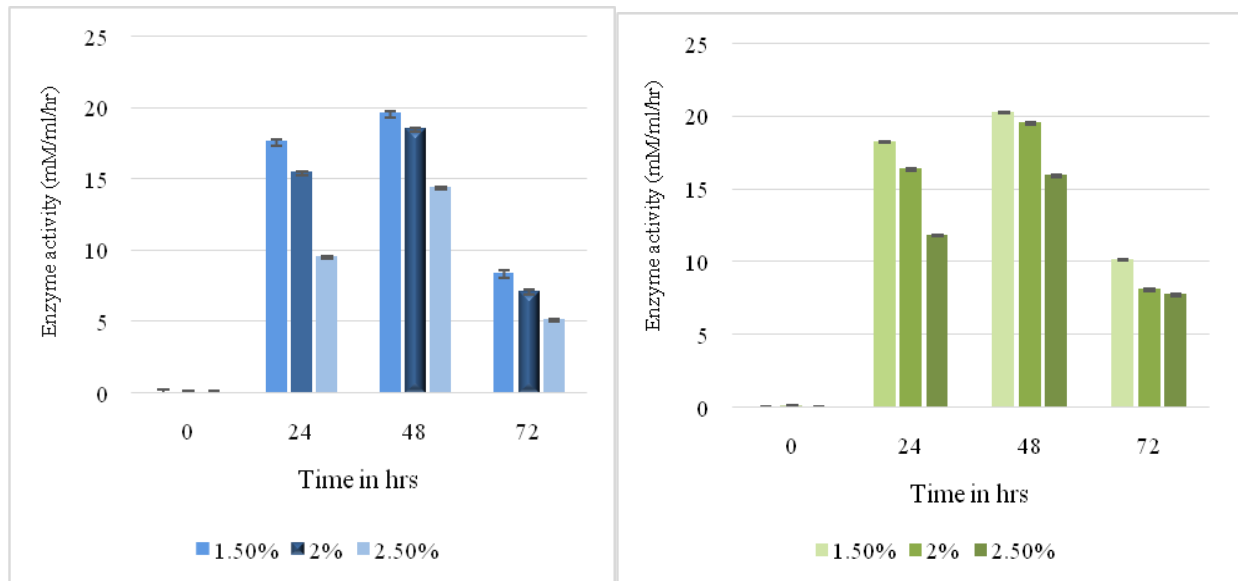


Fig.3 Effect of cell concentration on enzyme activity. **Fig.4** Effect of enzyme concentration on enzyme activity

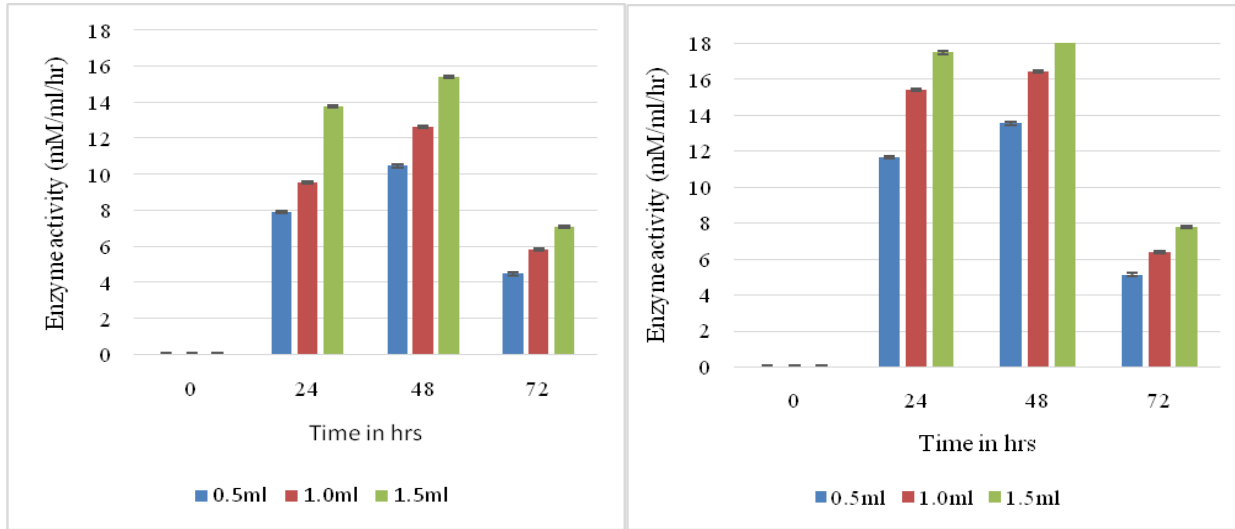


Fig.5 Effect of pH on enzyme activity

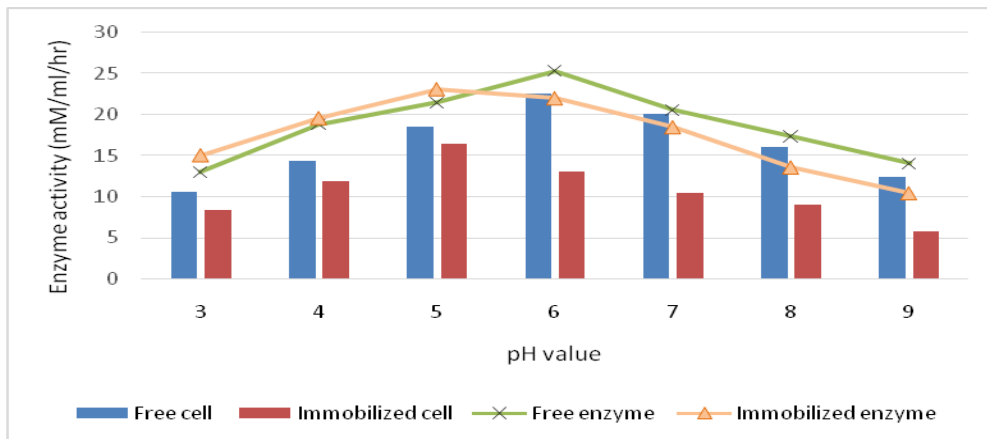


Fig.6 Effect of temperature on enzyme activity

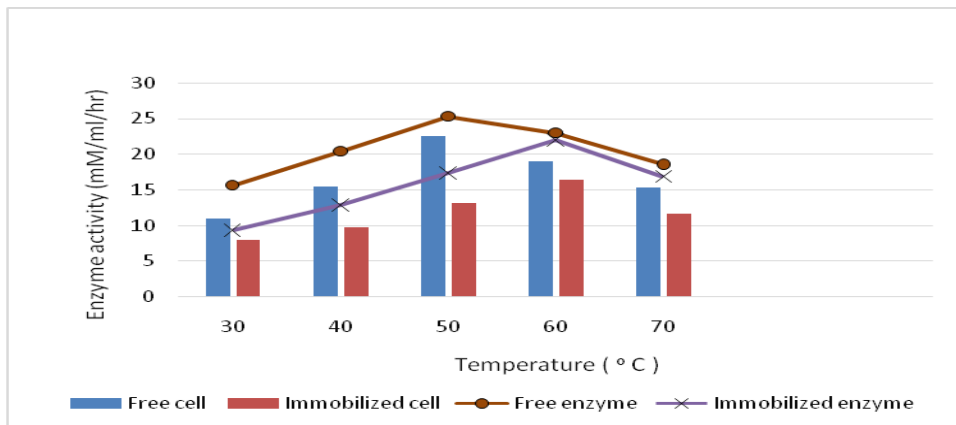
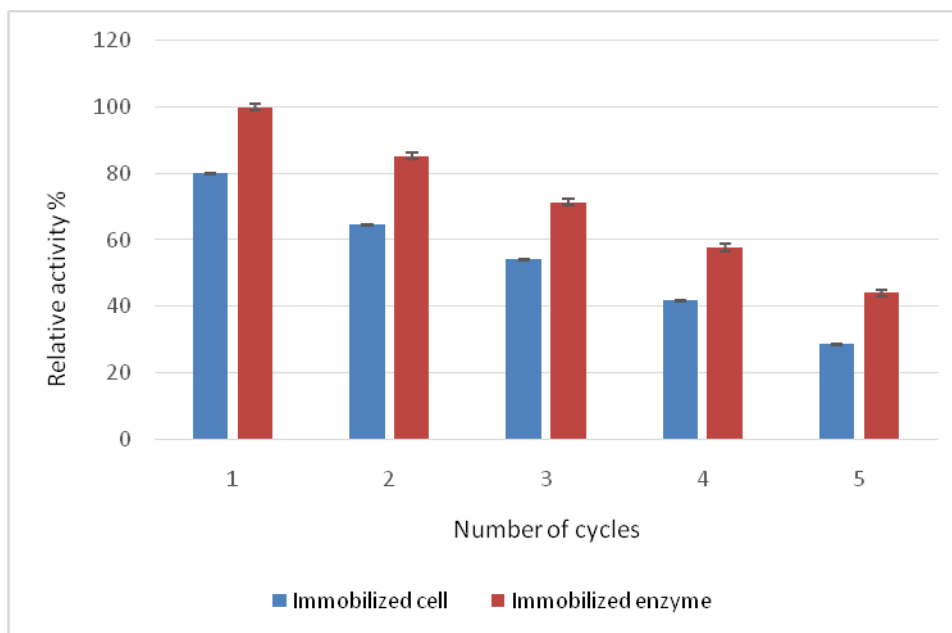


Fig.7 Reusability of immobilized cells and enzyme



Effect of gel concentration on enzyme activity

It is been reported that the yield of immobilized enzyme depends on the concentration of sodium alginate. Various concentrations of sodium alginate were used to prepare beads. The yield was found to be the highest at 1.5% concentration of sodium alginate (Table 3). The activity of immobilized enzyme (Fig. 2 and 3) was high as compared to immobilized cells by 23.51% (Fig. 1). The activity of immobilized enzyme was found to be equal when compared to the work done by Mohammed Abdu Al-ZaZae *et al.*, (2011).

Effect of cell concentration and enzyme concentration on enzyme activity

It was studied that the activity of enzyme and cell depends upon its concentration. The activity of enzyme (Fig. 4) and cell (Fig. 3) was observed to be high i.e., 20.41mM/ml/hr and 15.41mM/ml/hr respectively at 1.5ml of their concentration when incubated for 48 hrs

and decreased on further incubation. The activity of enzyme was found to be high as compared to cell by 33.3% (Table 4).

Effect of pH on enzyme activity

The optimum pH for free cell and free enzyme was observed to be 6.0. After immobilization the optimum pH decreased to 5.0. In high alkaline and acidic conditions immobilized cells and enzyme did not show expected activity (Fig. 5). When compared with the studies done by Basabrani Devi *et al.*, (2012), the optimum pH of immobilized cells and enzyme was found to be more.

Effect of temperature on enzyme activity

The optimum temperature for free cell and free enzyme was observed to be 50⁰ C, after immobilization the optimum temperature increased by 10⁰ C (Fig. 6). When compared with the studies done by EsawyMona Abdeltawab *et al.*, (2014), the optimum temperature of immobilized cells and enzyme was found to be high.

Productivity of immobilized enzyme and immobilized cells in different bioreactors

The activity of immobilized enzyme was high as compared to immobilized cells by 23.51%. The productivity was then checked using different bioreactors. The enzyme activity was found to be highest in the Air Lift Reactor then Shake Flask and lowest in Column Reactor. The enzyme activity of the immobilized enzyme in the Air Lift Reactor was found to be 20.99% more when compared with the activity in the Shake Flask. Citric acid production from 20% starch using free enzyme and free cell was found to be 76% and 68% respectively after 8 days of fermentation. Citric acid production by immobilized enzyme in airlift reactor was found to be 38.63% more when compared to shake flask (Table 5).

Reusability of immobilized cells and immobilized enzyme

The main advantage of immobilization is its reusability. Its use is also cost effective in industries. The immobilized cells or the enzyme can be reused after separation by filtration. The reusability of the immobilized cells and enzyme was studied up to 5 cycles. It was found that the activity and reusability of immobilized α -amylase was high as compare to that of immobilized cells producing α -amylase. The activity of immobilized enzyme decreased to 43% after 5th cycle which was low as compared to the work done by Talekar Sachin *et al.*, (2012). The activity of immobilized cells decreased to 28% after 5th cycle as shown in the figure 6.

The enzyme activity of free enzyme was high as compared to that of free cell. Highest activity was observed at 48 hrs of incubation and declined then after. The activity of immobilized enzyme was high as compared to that of immobilized cells by 23.51% at 1.5%

of gel concentration. The enzyme activity increased with the increase in cell and enzyme concentration. It was high at 1.5ml of cell and enzyme concentration. Immobilized enzyme and cells showed great stability at high temperature i.e., 60^o C and pH 5.0. The productivity of immobilized cells and enzyme was high in Air lift reactor than in shake flask and column reactor. The activity of immobilized cells and enzyme decreased upto 28% till 5th cycle. Citric acid production by immobilized enzyme in airlift reactor was found to be 38.63% more when compared to shake flask. The optimization of bioreactor parameters are still to be worked on for efficient utilization of the unconventional method for production of citric acid from starch for further scale-up and pilot plant studies.

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