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CHARACTERIZATION OF PURIFIED FREE AND IMMOBILIZED α- AMYLASE ENZYME PRODUCED BY *BACILLUS MEGATERIUM* KLMI 4

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ABSTRACT
The present microbe is also a mesophilic as indicated by its activities based on temperature levels. The ambient temperature plays a great role in the growth and any of the activities of any microbe. Lower temperature levels (21 to 29 ^{0}C) did not support the enzyme activity. Slightly lower activity was observed in case of immobilized form at 21 and 37^0 C than that in free form of the enzyme. While peak activity was noticed at 37^0 C in free form, peak activity occurred at 41^0 C in case of immobilized form. Thus, a shift in temperature-related enzyme activity appears to have set in due to immobilization. Both forms of the enzyme exhibited nil activity at *p*H 5.0. From *p*H 6.0 onwards, the relative activity of immobilized form was certainly higher than that of the free form and in both forms peak relative activity of 100% was recorded at *p*H 8.0. The relative activity decreased sharply to 25% in case of free form of the enzyme, while it was very near to 100% at *p*H 9.0 and decreased to only 65% at *p*H 10.0, thus indicating an improved tolerance to higher *p*H levels due to immobilization. Reusability of enzyme in repeated batch fermentations: > 60% activity retained in the 7th cycle and Storage stability ~ 70% residual activity on 25th day of storage. The optimum substrate concentration for purified and free form of enzyme was found to be 1.0%. While, purified and immobilized form of enzyme utilizes 1.2% of substrate concentration with 100% its relative activity. K_m =0.66, V_{max}= 166.66, values of purified free α-amylase and Km =2.7, V max =142.85, values of purified immobilised α-amylase were calculated from Lineweaver- Burke plot.

KEYWORDS: Alpha-amylase, Immobilization, Characterization.

INTRODUCTION
Amylases are starch hydrolyzing enzymes and are widely distributed throughout the microbial, plant and animal kingdoms. They degrade starch and related polymers yielding products that are characteristics of individual amylolytic enzymes. Initially, the term 'amylase' was used to designate enzymes capable of hydrolyzing α-1, 4-glycosidic bonds of amylose, amylopectin, glycogen and their degradation products.

Enzyme immobilization is an important technique for economizing the fermentation process to garner a desired end product. Immobilization of the enzyme in a matrix provides strength, increased stability, increased protection from ambient factors, reusability (repeated reuse of the used immobilized enzyme), improved storage, etc.

MATERIALS AND METHODS
Immobilization of Purified Enzyme
The purified enzyme obtained after ion exchange chromatography wit 150 IU/ml enzyme activity was entrapped on sodium alginate as per Naganagouda and Mulimani (2006) with 2% calcium chloride and 3% sodium alginate.

Estimation of Enzyme Immobilized in the Alginate Beads
Entrapment of the enzyme into the beads was determined by taking into consideration the difference between the activity of the free enzyme and that remaining in the washed water as well as the filtered calcium chloride solution in the following manner:

$$\text{Immobilization yield}(\%) = \frac{(\text{Activity of added enzyme}) - (\text{Activity of incubated solution} + \text{Activity of washed buffer})}{\text{Activity of added enzyme}} \times 100$$

Physico-Chemical Properties of the Free and Immobilized Enzyme
Various physico-chemical properties of the free and immobilized enzyme like temperature and *p*H tolerance, reusability, storage stability and kinetic measurement were studied. The relative activity of the enzyme entrapped in the alginate beads was considered to be hundred per cent in all these studies.

1. Thermo-stability of the Enzyme

The influence of the temperature on the free and immobilized enzyme was studied in the following manner. The free enzyme (1.0 ml) and 1.0 g beads with immobilized was added to tris-HCl buffer (*p*H 8.0) with 1.5% starch and incubated for 30 min at different temperature levels (21^0 to 61^0 C) in constant temperature water baths and then assayed for enzyme activity employing the DNS method (Millers 1959). The results are presented in terms of relative activity.

2. Enzyme Stability at Different *p*H

The free (1 ml) as well as immobilized enzyme (1.0 g beads) were separately added to buffers with 1.5% starch for studying the tolerance of both forms of the enzyme to different *p*H levels (*p*H 5.0 to 10.0). Different buffers were used for different *p*H levels: sodium citrate buffer for *p*H 5.0, sodium phosphate buffer for *p*H 6.0 to 8.0, glycine-sodium hydroxide buffer for *p*H 9.0 and *p*H 10.0. The two preparations (free enzyme and enzyme embedded in the alginate beads) with different buffers were incubated for 30 min at 37^0 C and thereafter the enzyme activity was assayed as detailed above. The results are presented in terms of relative activity.

3. Reusability of Immobilized Enzyme

The Erlenmeyer flasks (50 ml) in triplicate with 10 ml tris-HCl buffer with 1.5% starch were added with immobilized beads and incubated at 37^0 C for 30 min. Thereafter, the supernatant solution was assayed for amylase activity as per the DNS method briefed earlier. This constituted the first cycle of use of the immobilized beads. Then, the supernatant was drained off and the beads were washed thrice with the buffer. The beads were subjected to fermentation of the fresh reaction mixture as in the first cycle and enzyme activity was assayed by considering the enzyme activity in the first cycle as 100%. In this way, the immobilized beads were used repeatedly and at the end of each cycle, enzyme was assayed.

4. Storage Stability of the Free and Immobilized Enzyme

It was considered worthwhile to estimate the length of time the enzyme, either in free or immobilized condition, can be stored. The two forms of the enzyme were stored in tris-HCl buffer (*p*H 8.0) at 4^0 C separately over a span of 30 days. Every 5 days the residual activity of the enzyme was assayed as mentioned above taking into consideration its initial activity in each form, the results being expressed as percentage of respective initial activity.

5. Kinetic Analysis of Free and Immobilized forms of α-amylase

Kinetic parameters of free and immobilized forms of α-amylase were estimated by measuring the initial reaction rates using starch concentrations in the range of 0.1-1.5% at pre-determined optimum *p*H and temperature of each form. For both forms of enzyme (free and immobilized), the Michaelis-Menton kinetic behaviour was observed. K_m and V_{max} values of free and immobilized α-amylase were calculated from Lineweaver-Burke plot.

RESULT

Estimation of Enzyme Immobilized in the Alginate Beads

As per the calculations made as detailed earlier, when the purified enzyme with 150 IU/ml/min activity was immobilized, the activity of the immobilized enzyme was observed to be 125 IU/ml/min. Hence, the immobilization yield was observed to be 83.33%.

Physico-Chemical Properties of the Free and Immobilized Enzyme

Various physico-chemical properties of the free and immobilized enzyme like temperature and *p*H tolerance, reusability, storage stability and kinetic measurement were studied. The relative activity of the enzyme entrapped in the alginate beads was considered to be hundred per cent in all these studies.

1. Thermo-Stability of Free and Immobilized α-Amylase

Lower temperature levels (21^0 to 29^0 C) did not support the enzyme activity, with 12.5 to 40 IU/min in both forms. Slightly lower activity was observed in case of immobilized form at 21 and 37^0 C than that in free form of the enzyme. At temperature levels higher than 37^0 C, i.e. at 41 and 45^0 C, the picture was reversed. While peak activity was noticed at 37^0 C in free form, peak activity was noticed at 41^0 C with immobilized form. Apart from this, while free enzyme activity was reduced by 43% at 45^0 C, it had marginally reduced by 7% in case of the immobilized form. Thus, a shift in temperature-related enzyme activity appears to have set in due to immobilization (Fig. 1).

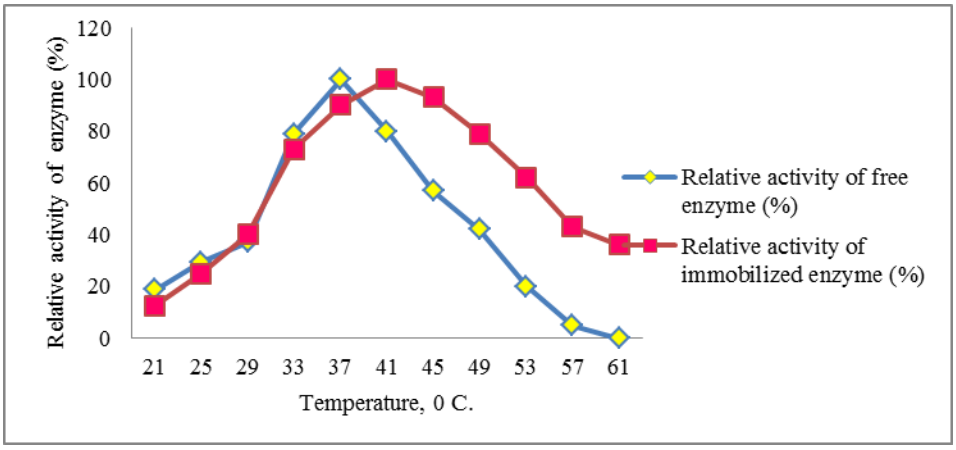

Fig. 1: Thermo-Stability of the Free and Immobilized Enzyme.

2. Influence of *p*H on free and immobilized enzyme

The results are presented in Fig. 2. Both forms of the enzyme exhibited nil activity at *p*H 5.0. From *p*H 6.0 onwards, the relative activity of immobilized form was certainly higher than that of the free form and in both forms peak relative activity of 100% was recorded at *p*H 8.0. The relative activity decreased sharply to 25% in case of free form of the enzyme, while it was very near to 100% at *p*H 9.0 and decreased to only 65% at *p*H 10.0, thus indicating an improved tolerance to higher *p*H levels due to immobilization.

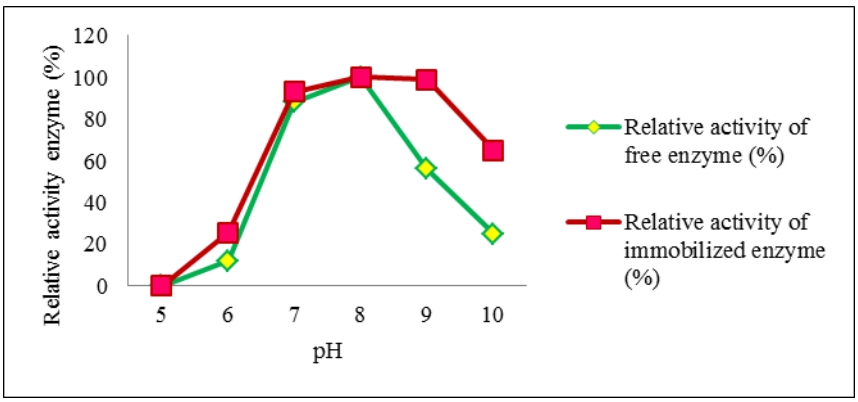

Fig. 2: Enzyme stability influenced by *pH*.

3. Reusability of Immobilized Enzyme

The activity of the immobilized enzyme in the first use (i.e. '0' cycle) was observed to be 125 IU/ml and this value has been considered to be 100% in terms of relative activity. Same activity was recorded in the subsequent first reuse (cycle 1), i.e. 100% relative activity. Thereafter, with every reuse the enzyme activity started to decrease gradually, reaching 52% relative activity at the end of 8th cycle. In the subsequent reuses, it decreased sharply to 20% by the end of 11th cycle. The results are presented in Fig.3.

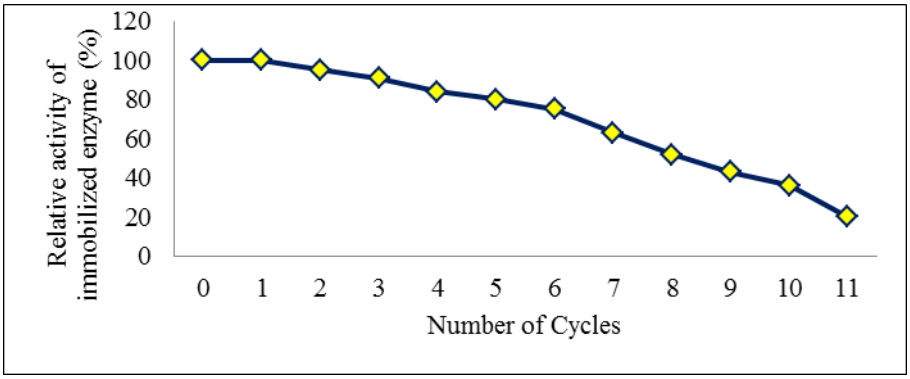

Fig. 3: Reusability of the immobilized enzyme.

4. Storage stability of the Free and Immobilized α-amylase

The results pertaining to the storage suitability of the immobilized enzyme are presented in Fig. 4.

The results indicate that the enzyme retained 100% residual activity upto the 10th day of storage. In the subsequent samplings, the residual activity continued to drop to 40% at the end of 30th day. Thereafter, the study was discontinued. On the other hand, the free enzyme continued to decrease from the 1st sampling (i.e., after 5 days of storage) and thereafter the activity of the free enzyme decreased sharply decreased to 10% on the last (30th day) sampling. Thus, the immobilized enzyme can be stored for 25 days since the enzyme retains 68% of its activity.

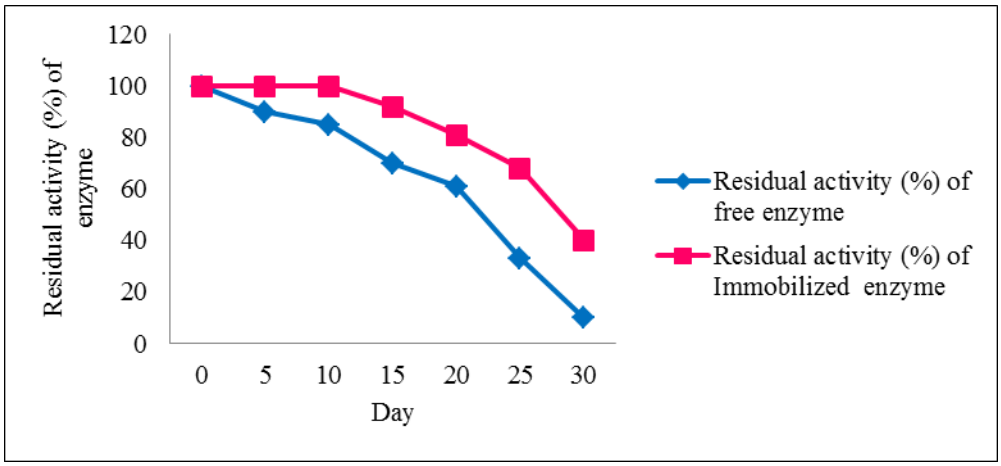

Fig. 4: Storage stability of the free and immobilized enzyme.

5. Kinetic Analysis of Free and Immobilized forms of α-amylase

In the studies pertaining to the kinetic analysis of the free and immobilized enzyme, the optimum substrate concentration for each form was initially determined. The results are presented in Fig. 5.

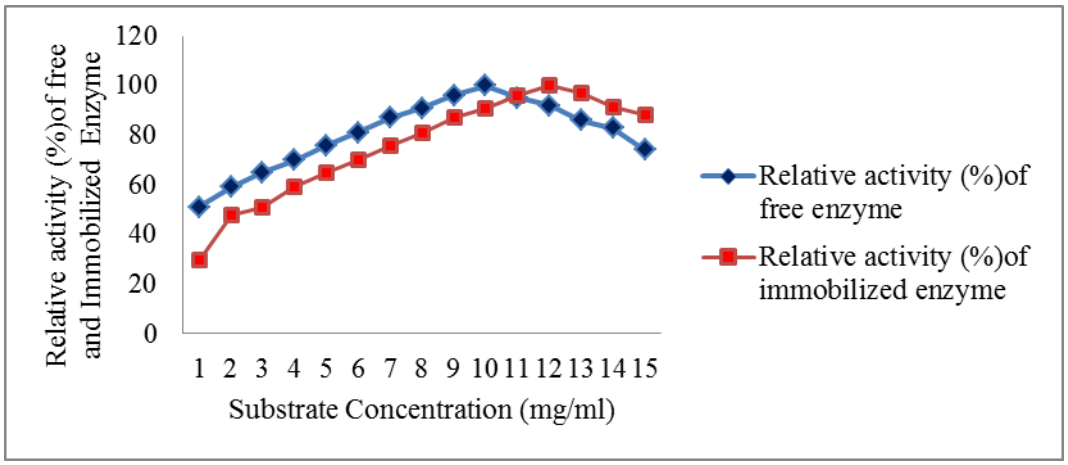

Fig. 5: Influence of substrate on relative activity of free and immobilized enzyme.

In the controls (with free enzyme), the enzyme activity increased with increase in substrate concentration and maximum enzyme activity of 150 IU/ml was observed at substrate concentration of 10 mg/ml substrate (starch). This was considered as the 100% relative activity in the further studies. Thereafter, the enzyme activity decreased gradually.

In case of the immobilized enzyme, the enzyme activity was 37.7 IU/ml at substrate concentration of 1 mg/l and thereafter it gradually increased to 125 IU/ml at substrate concentration of 12 mg/ml. At substrate concentrations above 12 mg/ml the enzyme activity decreased sharply. The enzyme activity at 12 mg/ml (i.e., 125 IU/ml) was considered as 100% relative activity for the immobilized enzyme.

Kinetic Analysis of Free Enzyme

The results of kinetic studies of the free enzyme (Fig. 6) revealed that the K_m value to be 0.66 and its V_{max} value to be 166.66.

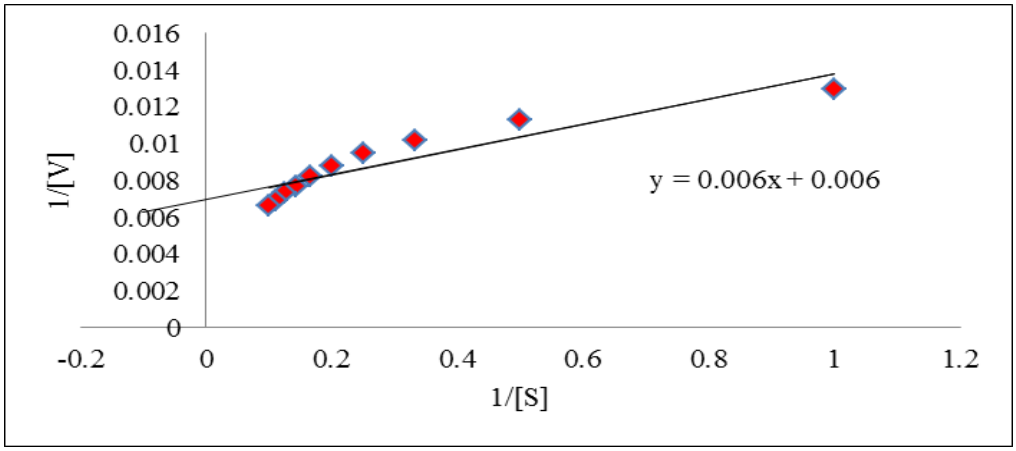

Fig. 6: Kinetic analysis of free enzyme.

Kinetic Analysis of Immobilized Enzyme

The results of kinetic studies of the immobilized enzyme (Fig. 7) revealed that the K_m value to be 2.70 and its V_{max} value to be 142.85.

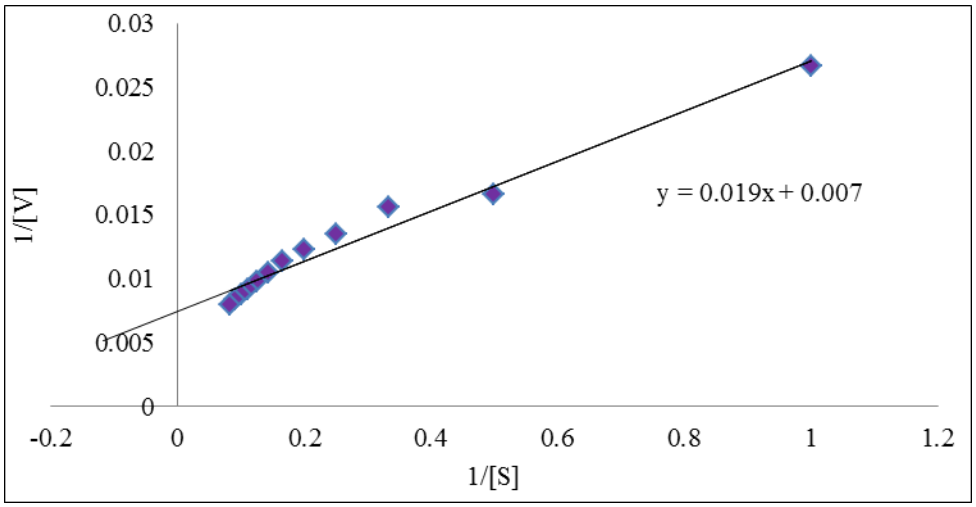

Fig. 7: Kinetic analysis of immobilized enzyme.

Table 1: Enzyme immobilization and characterization.

Source	Matrix	K_m Free Immo.	V_{max} Free Immo.	Reference
B. subtilis	AS-Alumina, DEAE cellul.	2.72 4.0 – 4.76	28.6 4.0 – 5.3	Abdel-Naby *et al.* (1998)
B. subtilis	Na-alginate, chitin, sepha-dex, dowex	8.3 16.6	10.0 30.3	El-Banna *et al.* (2007)
B. acidocald - arius	Glass beads/ Cationic. exch. resin	0.86 1.05 0.86 0.91	144.1 127.6 144.1 125.8	Ahmed *et al.* (2008)
A. carbonarius	Silica gel	3.54 2.13 – 3.54	23.8 20.01 – 37.90	Nwagu *et al.* (2011)
F. solani	Na-alginate	27.47 18.52	5.28 1.23	Kumar *et al.* (2012)
B. amylolique-faciens	Silica gel/phy. gel	14.69 23.55	1.31 1.14	Dragomirescu *et al.* (2012)
B. amylolique-faciens	CLEA	2.748 0.3245	0.174 0.179	Talekar *et al.* (2012)

DISCUSSION

Upon immobilization of the enzyme in the alginate beads, a loss of nearly 17% in its activity has been observed. However, some workers have reported increases in enzyme activity upon immobilization when compared to that of the free enzyme to the tune of 14% with *Pseudomonas* sp. (Shobha *et al.*, 2001), 9% with *B. amyloliquefaciens* (Dincbas and Demirkan, 2010) and

36% with *Pontibacillus chungwhensis* (Mageshwari *et al.*, 2012). On the other hand, decreases of 18% (Devi *et al.*, 2012) and 45% (Dragomirescu *et al.*, 2012) in α-amylase activity of *Bacillus* sp. and *B. amyloliquefaciens*, respectively, upon immobilization have been reported. Hence, the present observation of 17% decrease in α-amylase activity upon immobilization in case *B. megaterium* KLMI4 is in line with the observations of Devi *et al.* (2012) and Dragomirescu *et al.* (2012). The enzyme tends to lose some of its activity while being trapped in the alginate beads. The loss may be reflected in some enzyme being washed away in the wash waters of the beads.

In the present study, it has been observed that immobilization in alginate beads tended to impart more stability to the enzyme. The free enzyme had its temperature optima at 37^0 C while for the immobilized enzyme it was observed to be 41^0 C. The relative activities of the free and immobilized forms at 61^0 C were 0 and 36%, respectively. Such a shift in temperature optima has been observed in different species and strains of *Bacillus* and other bacterial species too as observed by various earlier workers (El-Banna *et al.*, 2007; Ahmed *et al.*, 2008; Riaz *et al.*, 2009; Demirkan *et al.*, 2011; Talekar *et al.*, 2012; Mouafi *et al.*, 2016). The present observation is in line with the observations of these researchers. Dragonmirescu *et al.* (2012) have reported a decrease in temperature optima as a result of immobilisation of the enzyme.

In the same way, immobilization in alginate beads tended to give the enzyme more tolerance to ambient *p*H conditions. The *p*H stability studies were carried out in the range of 5.0 to 10.0. Generally, the immobilized enzyme exhibited more relative activity than the free form in both the acidic and alkaline *p*H conditions. Though the *p*H optima for both were 8.0, the immobilized form exhibited a relative activity of 99% at *p*H 9.0 and 65% at *p*H 10.0, while the free form exhibited relative activities of only 56% at *p*H 9.0 and it dropped to 0% at *p*H 10.0. Thus, though *p*H optima were not altered by alginate immobilization it did influence better activity of the enzyme at higher alkaline *p*H levels. Some researchers have reported that *p*H optima in enzymes from the test organisms remained the same (Ahmed *et al.*, 2008; Riaz *et al.*, 2015; Bal *et al.*, 2016). Our observations are in agreement with those of these workers. However, Devi *et al.*, (2012) noticed a shift in *p*H optima from acidic to neutral levels (6.0 to 7.0). Similarly, neutral *p*H optima to acidic optima has been reported by El-Banna *et al.* (2007), Dragomirescu *et al.* (2012), Talekar *et al.* (2012) and Mouafi *et al.* (2016), while Abdel-Naby *et al.* (1998) have reported acidic to more acidic *p*H optima as a result of immobilization of the enzyme.

In the reusability studies, it was observed that the immobilized enzyme retained its original activity in the first cycle. Thereafter the relative activity gradually decreased coming down to 52% in the 8^{th} cycle. In the 11^{th} cycle, it dropped to 20%. Thus, the immobilized enzyme can be reused 8 times. Such reusability of the immobilized enzyme has been reported by several workers: 3 cycles (Riaz *et al.*, 2015), 4 cycles (Talekar *et al.*, 2012), 5-7 cycles (Gangadhharan *et al.*, 2008) and 10 cycles (Shobha *et al.*, 2001; Devi *et al.*, 2012).

The storability studies indicate that the immobilized enzyme can be stored for 25 days when 68% of its relative activity is retained. Such storage studies by other workers appear to be rather limited.

On the whole, it is safe to conclude that immobilization of the enzyme in the alginate beads tends to impart more tolerance to ambient conditions, increases its reusability as well as its storage stability.

Several researchers have successfully immobilized α-amylase in different matrices. In the present study, sodium alginate has been employed to immobilize the purified enzyme of *B. megaterium* KLMI4.

From the kinetic studies, the K_m and V_{max} values of the free enzyme are observed to be 0.66 mg/ml and 166.66±0.02 µmole/min, while those of the immobilized enzyme are 2.70 mg/ml and 142.85±0.02 µmole/min for *B. megaterium* KLMI4 in the present study. Different workers have reported the K_m and V_{max} values for both the free and immobilized α-amylases, as mentioned in the below mentioned Table. 1.

The relation between the substrate concentration and the rate of reaction can be described by the Michaelis-Menten equation. The immobilized enzyme showed higher K_m value than that of the free enzyme because of the enzyme concentration gradient. Generally, K_m of an immobilized enzyme is higher than that of the free enzyme due to lesser availability of substrate to the active site of the enzyme, with some exceptions (above Table.1). The present study appears to be first comparative kinetic analysis of the free and immobilized enzyme of any strain of *B. megaterium*.

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