Maltose deterioration approach: Catalytic behavior optimization and stability profile of maltase from *Bacillus licheniformis* KIBGE-IB4

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A B S T R A C T

Maltase is an economically valuable enzyme that is used to catalyze the hydrolytic process of maltose and yields D-glucose as a product. In this study, the catalytic behavior of maltase was optimized under various physicochemical condition. Results indicated that bacterial maltase exhibited maximum catalytic activity at 45 °C and pH-6.5 after 5.0 min. It presented greater stability within 0.1 M K_2HPO_4 buffer having pH-6.5 and showed 100 % activity even after 1.0 h. It retained 83.6 % and 45.0 % activity at 40 °C after 1.0 and 3.0 h, respectively. The enzyme retained 90.0 % activity at -20 °C even after 60 days. The molecular weight of enzyme was deduced to be 157.2 kDa as calculated using polyacrylamide gel electrophoresis (PAGE) and zymography. It was concluded that the characterized maltase has notable stability profile with reference to temperature, pH and other reaction conditions which anticipates its utilization in various starch and maltose hydrolyzing processes for the synthesis of glucose.

1. Introduction

Maltase [E.C 3.2.1.20] is included among amylolytic enzymes that are involve in the cleavage of α-(1→4) glycosidic linkages present in maltose and produces D-glucose as an end product. Alpha amylase depolymerizes the starch polymer into maltose which is further hydrolyzed into glucose molecules by the catalytic action of maltase [1–3]. The synergistic action of several amylolytic enzymes are responsible to completely deteriorate the maltose structure [4,5]. Maltase is produced by different microbial species intracellularly, extracellularly or as a cell bound form and plays a pivotal role in various biological systems. It is extensively used in food processing, alcohol production, different oligosaccharides and glucosides production, pharmaceuticals and fine chemicals production [6–13]. Myozyme and lumizyme are maltase based commercialized

products that are available in the world market. Clinically, maltase has been used for the treatment of pomp disease [14].

Bacillus licheniformis is widely distributed organism in nature. It is a gram positive, spore forming, aerobic and saprophytic bacterium. It is extensively utilized in many industrial processes for the production of wide range of useful products such as enzymes, antibiotics, fine chemical and consumer products [15]. These bacterial isolates have the ability of producing and releasing a cocktail of extracellular hydrolytic enzymes as they have strong potential to degrade several commercially important substrates into valuable products and easily cultivate on a wide range of nutrients. Due to aforementioned advantages, *B. licheniformis* has become a very important microorganism for several biotechnological process [16,17].

In this study, an extracellularly synthesized maltase from *B. licheniformis* KIBGE-IB4 was partially purified and characterized in terms of its catalytic behavior and stability. Also, the impact of various metal ions, detergents and organic solvents was examined on the catalytic activity of enzyme. The thermal and storage stability of the enzyme was also monitored at various temperatures such as 4 °C, 30 °C and -20 °C for 02 months for prospective industrial application.

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2. Materials and methods

2.1. Production of extracellular maltase from B. licheniformis KIBGE-IB4

Wheat starch was used as a sole carbon source to induce the previously isolated culture *B. licheniformis* KIBGE-IB4 for the synthesis of maltase [18]. A fermentation medium that contained (%): wheat starch, 2.5; peptone, 1.0; yeast extract, 0.2; meat extract, 0.4; K2HPO4, 0.3 and KH2PO4, 0.1 with pH-7.0 was used for enzyme production and incubated with bacterial isolate at 37 °C for 48 h [19]

2.2. Enrichment of maltase

Salting in and salting out method was used for enrichment of maltase. Therefore, ammonium sulphate was used for precipitation of protein using stepwise gradient precipitation technique from 10 to 60 % saturation. The precipitates of protein were collected after 18 h of saturation by centrifugation at 40,248 x *g* for 10.0 min. at 4 °C. The formed precipitates were then re-dissolved in a 100 mM K_2HPO_4 buffer of pH-6.5. After enrichment, the salts were removed from protein by performing dialysis against the same buffer overnight. These dialyzed precipitates were then used for further characterization.

2.3. Maltase activity assay

The catalytic activity of maltase was measured using GOD-PAP method using glucose as a reference sugar and maltose as a substrate [20,21]. One unit of maltase was defined as *"the amount of enzyme that is capable to liberate 1.0 μmol of glucose per minute under standard assay conditions"*.

2.4. Total protein quantification

The concentration of total protein was measured through Lowry's method and bovine serum albumin (BSA) was used as a reference protein [22].

2.5. Effect of pH on the catalytic behavior of maltase

Different buffers were evaluated for selection of suitable buffer as well as pH for enzyme substrate reaction. These buffers include acetate (pH 5.0–6.0), citrate (pH 5.0–6.0) and potassium phosphate (pH 7.0–8.0). After selecting the buffer, pH of that buffer was varied from 5.0–8.0 for optimization of pH while keeping all other conditions constant.

2.6. pH stability profile

The pH stability of maltase was analyzed by pre-incubating the enzyme without substrate in different pH environment (5.0–8.0) for 1.0 h. The enzyme containing aliquots were retrieved and catalytic activity was monitored (after adjusting the pH of all aliquots to optimum) in order to examine the stability profile of the enzyme for potential industrial use. The buffers used for this experiment were acetate buffer (pH-5.0–6.0) and potassium phosphate buffer (pH-6.5–8.0).

2.7. Effect of temperature on the catalytic behavior of maltase and its stability profile

The effect of reaction temperature on maltase activity was examined by performing the enzyme assay at different temperatures ranging from 30 °C to 60 °C. Maltase was further characterized on the basis of its thermal profiling by keeping the enzyme at different temperatures ranging from 40.0 °C to 60.0 °C for different time intervals (30.0–180.0 min.). After every 30.0 min., enzymatic activity was carried out and percent residual activity was measured.

2.8. Storage stability of maltase

Different storage temperatures (4 °C, 30 °C and -20 °C) were evaluated to examine the storage stability of maltase for 60 days. After every 10 days, the catalytic activity of enzyme was measured according to the standard assay procedure.

2.9. Substrate specificity of maltase

To observe the substrate specificity, partially purified maltase was allowed to react with different substrates including maltose, starch, sucrose and lactose at optimum pH and temperature.

2.10. Kinetic parameters of maltase

The influence of substrate concentration was also studied by performing the maltase assay at various concentration of maltose ranging from 2.5−50 mM in K_2HPO_4 buffer (pH: 6.0) to evaluate Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}) using GraphPad Prism® 7.0 software which was utilized for the estimation of non-linear regression analysis.

2.11. Effect of metal ions on the catalytic behavior of maltase

The effect of metal ions was investigated by pre-incubating dialyzed maltase with chloride forms of various metal ions including Ba+2, Co+2, Mn + 2, Mg+2, Zn+2, Cu+2, Ca+2, Cs+2, Na+, K+, Ni+2, Hg+2, Fe+2 and Ag + ions. The solutions were prepared in three different concentrations (1.0, 5.0 and 10.0 mM) and incubated with enzyme in 1:1 ratio for 30.0 min. prior to enzyme assay at 37 °C. The percent relative activity was compared with the control (enriched enzyme without exposure to any metal ions).

2.12. Effect of surfactants on the catalytic behavior of maltase

The impact of different surfactants on maltase activity was determined by pre-incubating the partially purified enzyme with Triton X-100, SDS, Tween 80 and metal ion chelator EDTA at 37 °C for 30.0 min. Different concentrations (1.0, 5.0 and 10.0 mM) of all aforementioned surfactants were analyzed in this experiment. The enzyme without any pretreatment was considered as control with 100 % relative activity.

2.13. Effect of organic solvents on the catalytic behavior of maltase

The effect of different organic solvents (DMSO, ethanol, methanol, isopropanol, formaldehyde and chloroform) on the catalytic performance of maltase was investigated by pre-incubating the enzyme with different concentrations (1.0, 5.0 and 10.0 mM) of organic solvents at 37 °C for 30.0 min. and enzyme assay was performed.

2.14. Molecular weight estimation and In-situ electrophoresis of maltase

Molecular weight of maltase was determined by PAGE using bovine serum albumin (BSA) as a marker. The enzyme was mixed with the sample diluting buffer (1:1 ratio) and the protein with known marker was initially loaded in their respective wells. Reservoir buffer of pH: 8.8 was filled in electrophoresis chamber

and electrophoresis was performed at 80.0 mV. After this, the gel was stained with coomassie brilliant blue stain and kept overnight with constant stirring. The gel was destained using destaining solution until dark bands were appeared with a clear background. The molecular weight was measured using gel documentation system.

Zymography was performed as described by Pan et al. (1989) with slight modifications [23]. After performing electrophoresis, the gel was washed with double deionized water thrice and incubated with 7.20 g L-1 solution of maltose which was prepared in 100 mM K$_2$HPO$_4$ buffer (pH: 6.0) for 30.0 min at 45 °C. Then zymogram was removed and washed repeatedly with same buffer and kept in 2, 3, 5-Triphenyltetrazolium chloride solution (dissolved in 1.0 N NaOH) and heated for 2.0 min with continuous shaking until the pink bands appeared.

3. Results and discussion

In the current study, the maltase from *B. licheniformis* KIBGE-IB4 was successfully partially purified and used for characterization in order to improve its utility profile for prospective industrial use. Enrichment was performed using ammonium sulfate precipitation method and 40 % saturation of ammonium sulfate gave higher enzymatic yield in terms of specific activity (Table 1).

The pH of the enzyme-substrate reaction mixture is among the important parameters that cause significant impact on the catalytic activity and stability of enzyme. Therefore in this study, different pH ranges were used from 5.0 to 8.0 in order to detect the most suitable pH for maltase as well as to understand the pH stability profile of maltase. It was observed that as the pH of the reaction mixture increased, the catalytic activity of maltase also increased and reach to its maximum at pH-6.5 (Fig. 1a). Further increase in pH beyond optimum decreased the catalytic activity while less activity observed at pH-10. The enzyme contains amino acid side chains having −COOR and -NH2 groups that have potential to gain or lose H + ions. The pH of the surrounding environment have great impact on charges of these groups and as a result of this the structural configuration of the enzyme can be changed which either increases or decreases the catalytic activity. McWethyt and Hartman [24] reported similar kind of observations when maltase from *Bacillus brevis* was evaluated for its pH profile. Further, it was also noticed that increase in pH beyond optimum value also have negative impact on the catalytic activity and about 29.6 % loss in the relative activity was found at pH-7.0 (Fig. 1a). It has also been reported that most of the enzymes showed their catalytic activity at neutral pH. Any change in pH either in acidic or alkaline side causes loss in activity, structural stability and solubility of protein as a result of changes in the charge groups [25]. Cihan et al. [26] reported pH-6.8 as optimum pH for the activity of α-glucosidase produced from *Geobacillus sp.* A333 that is relatively similar to current results where pH 6.5 was selected as the optimum pH for maltase produced from *B. licheniformis* KIBGE- IB4.

Consequently, the stability of maltase at these pH values was also investigated by pre-incubating the enzyme with different pH buffers ranging from 5.0–8.0 for 1.0 h. It was found that the maltase was stable at pH-6.5 with retention of 100 % activity after 1.0 h and lost its stability up to 92.16 % and 54.85 % at pH-5.0 and 8.0, respectively (Fig. 1b). The pH stability curve can be compared with pH optimum curve in order to understand the reversible and irreversible effects of pH. The reversible effect is the protonation of the amino acids in the active center while charge alteration of structurally important amino acids mostly generate irreversible changes in the enzyme native structure [27]. The loss of catalytic activity of maltase under extreme pH values for example pH 5.0 and 8.0 demonstrated the irreversible effect of these pH on maltase activity.

The reaction temperature is another critical factor for evaluation of the enzyme activity. The temperature is responsible to increase the kinetic energy of enzyme which ultimately increases the collision frequency of enzyme and substrate. The catalytic activity of enzyme increases as the temperature increases up to its optimum temperature, beyond this point, the structural conformational of protein is disturbed due to increased kinetic energy of the molecules at higher temperature which eventually declines the catalytic performance of enzyme. Therefore, the effect of temperature on the activity of maltase was observed by varying the temperature of reaction mixture from 30 °C to 60 °C. It was noticed that the maltase activity increased as the temperature increased from 30 °C to 45 °C with maximum activity was attained at 45 °C (Fig. 1c). A 90.01 % reduction in activity was observed at 60 °C which might be due to increase in kinetic energy that causes cleavage of peptide bonds and ultimately alters the structural arrangement of enzyme molecule [27]. These results are very much similar to the previously reported findings of optimum temperature for catalytic activity of extracellular maltase from *Bacillus brevis* [24].

The stability of enzyme in response to different temperatures represents the capability of an enzyme to tolerate harsh industrial conditions. The thermophilic enzymes are also one of the major requirements to proceed different bioprocesses at commercial scale [28,29]. For determination of thermal tolerance, enzyme was kept at various temperatures (40 °C–60 °C) for 180.0 min. and the residual activity was calculated after every 30.0 min. (Fig. 1d). It was observed that the enzyme lost its catalytic activity as time increased. Maltase retained about 45 % and 25 % of its initial activity after 180.0 min. at 40 °C and 45 °C, respectively while complete loss of activity was observed at 50 °C after same time interval (Fig. 1d). Further, keeping maltase at 55 °C for 60.0 and 90.0 min. resulted in 94 % and 100 %, respectively loss of initial activity which showed that at this temperature enzyme was completely denatured. These results suggested that maltase can with stand 40 °C and 45 °C for longer time intervals as compared to other temperatures. The loss of maltase activity at high temperatures due to exposure for longer time might be due to the increase in kinetic energy of the molecules. As a result of which the three dimensional conformation of an enzyme can be disrupted at certain level due to cleavage of different attractive forces between amino acids which are accountable to maintain the native structure of enzyme.

Furthermore, maltase assay was performed in presence of different buffers in order to understand the activation and

Table 1
Purification steps, fold purification and percent recovery of maltase.

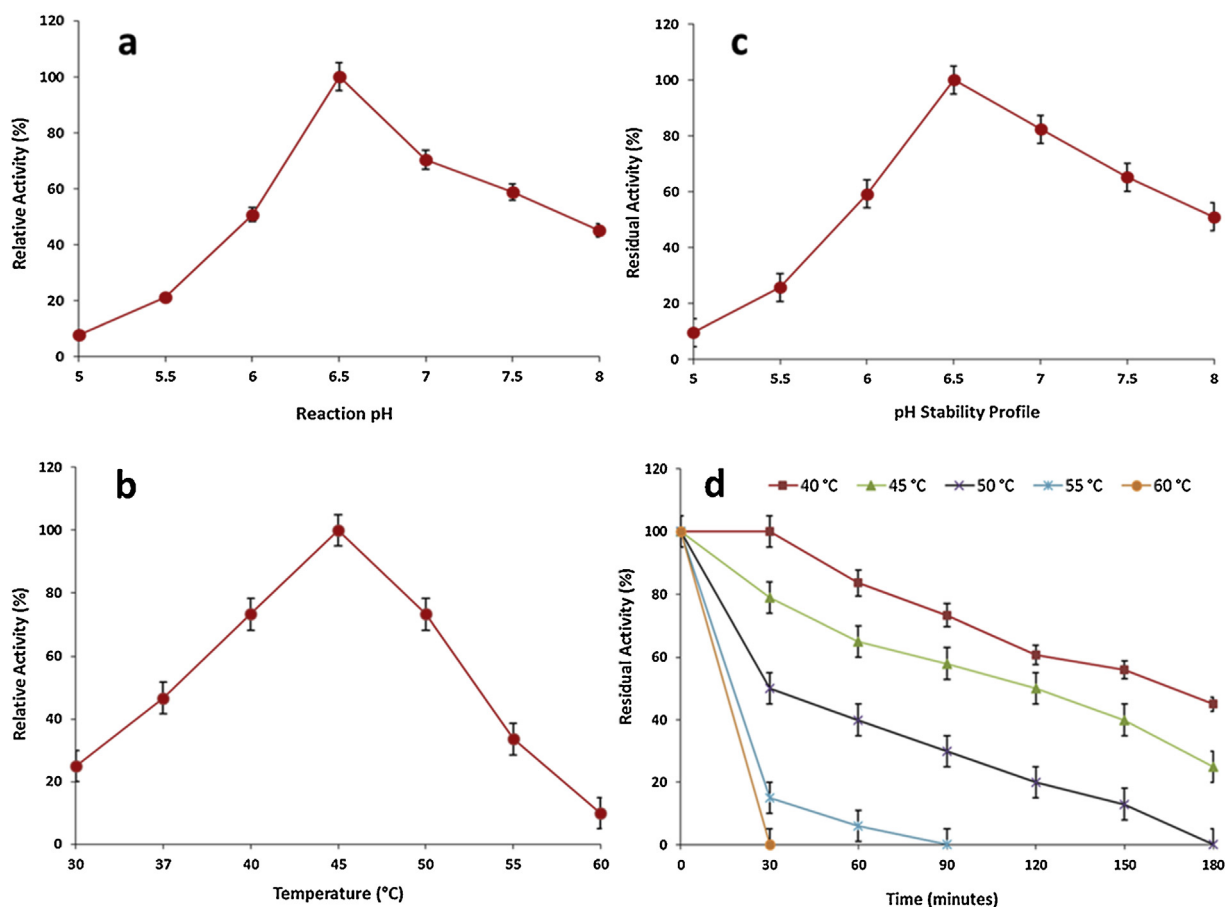| Samples	Volume (ml)	Enzyme Activity (U ml-1 min-1)	Total Activity (U)	Total Protein (mg ml-1)	Total Protein (g)	Specific Activity (U mg-1)	Fold Purification	Yield (%)
Crude enzyme	100	841.46	84146	7.34	734	114.64	1.0	100
Enzyme Precipitates (40%)	5.0	3032.55	15162.5	5.23	26.15	579.83	5.05	18.01
Dialyzed Precipitates	6.9	2965.50	20461.9	4.55	31.39	651.75	5.68	24.31

Fig. 1. Effect of various pH and temperature on the catalytic activity (a, b) and stability (c, d) of maltase.

deactivation phenomenon of enzyme. Results indicated that among different tested buffers, phosphate buffer was found to be the most suitable buffer for hydrolytic activity of maltase (Fig. 2a). Buffers are responsible to provide the stability to the enzyme's structure as it contain charged molecules which affects the binding of substrate with active site of enzyme [29].

Storage stability of an enzyme is a crucial factor when the enzyme catalytic behavior and its properties are optimized. Enzyme shelf life or storage stability denotes for how many days enzyme is capable of retaining its catalytic properties starting from its production to its utilization in industrial processes. During storage, enzymatic properties are gradually declining due to protein unfolding as a result of shifting enzyme filtrate into heating and cooling from ambient temperature [30,31]. In the current study, different temperatures like (-20.0 °C, 4.0 °C and 30.0 °C were used to determine the stability of enzyme for 60 days (Fig. 2b). The catalytic activity was measured after every 10 consecutive days. It was monitored that 68 % and 37 % activity of maltase was retained at 30.0 °C after 30.0 and 60.0 days, respectively. Moreover it was also observed that at -20 °C and 4 °C, maltase was relatively more stable and retained about 90 % and 52 % activity after 60.0 days. Lyer and Ananthanarayan [32] reported that this loss of enzymatic activity during storage is might be due to autolysis, unfolding and denaturation of protein structure.

The substrate specificity of maltase was also investigated by incubating enzyme with different substrates including maltose, starch, sucrose and lactose. Fig. 2c showed that hydrolyzing pattern of maltase towards maltose is more rapid as compared to other substrate (starch and sucrose) while no hydrolysis was observed in case of lactose. Similar pattern of hydrolysis was also previously reported for maltase from newly isolated *Geobacillus sp.* A333 [33].

The kinetic parameters (*Km* and *Vmax*) were calculated by using different concentrations of substrate (2.5 mM–50.0 mM). It was found that the *Km* value was 1.717 mM ml-1 (standard error: 0.052 mM ml-1) and *Vmax* was 8411.0 U g^{-1} (standard error: 213 U g^{-1}).

The catalytic activity of enzyme has been previously studied in the presence of several metal ions [33,34]. Some metal ions activate the hydrolytic activity of enzyme while others may act as inhibitors for different enzymes. The effect of different metal ions including Na+, K+, Ba+2, Co+2, Mn+2, Mg+2, Zn+2, Cu+2, Ca+2, Cs+2, Hg+2 and Fe+2 on the catalytic activity of maltase was studied (Table 2). The results suggested that the most of the metal ions such as Ba+2, Co+2, Mn+2, Mg+2, Zn+2, Cu+2, Ca+2, Cs+2, Na+, K + and Fe+2 stimulated the enzyme activity except nickel Ni+2 which showed 41.66 % loss in catalytic activity of enzyme. Mercuric chloride showed significant inhibitory effect on maltase. It has been previously reported that Hg+2 inactivates the enzyme by interacting either specifically with thiol group or non-specifically with a carboxyl group or histidine residues in the enzyme molecule [35]. When metal ions such as Cu+2 and Zn+2were pre-incubated with maltase for 60.0 min, 9.50 % and 29.61 % inhibition in activity was observed; whereas, in the presence of others divalent cations
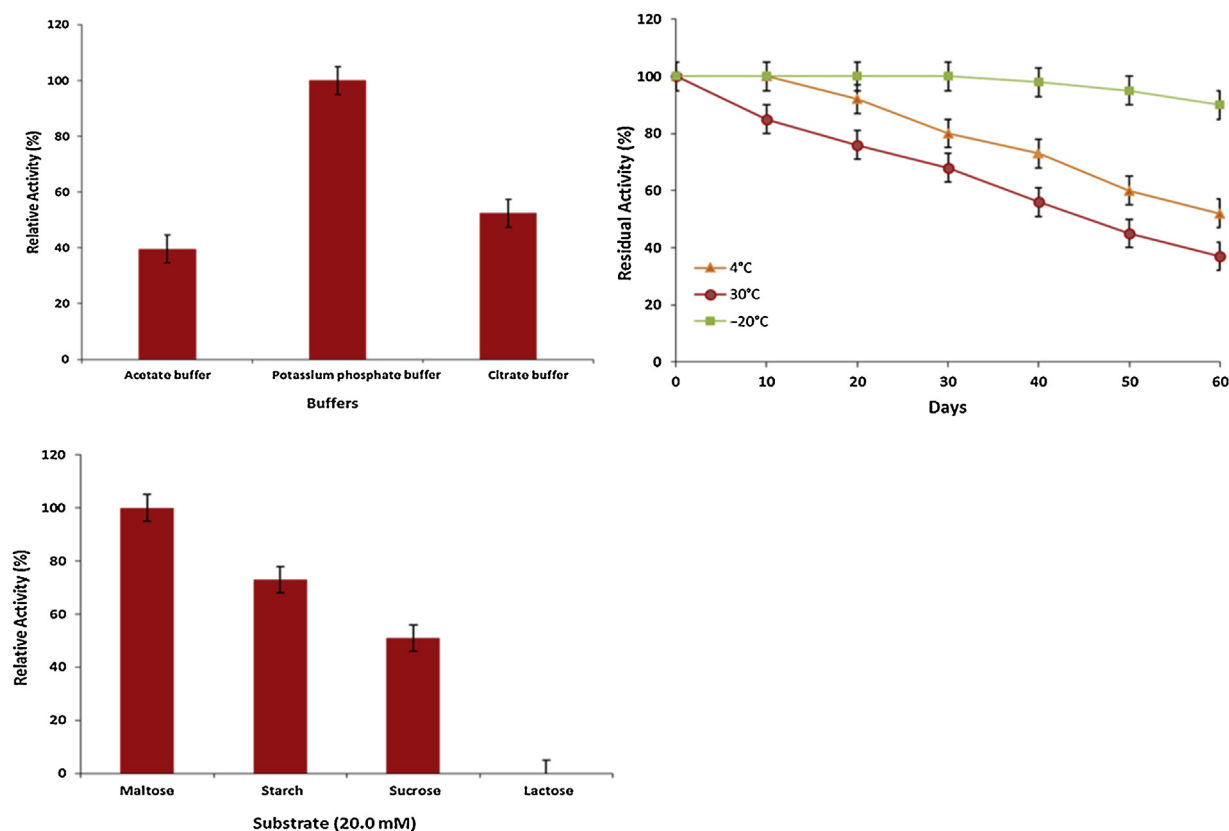
Fig. 2. Effect of different buffers (a); storage stability (b) and substrate specificity of maltase (c).

Table 2
Effect of metal ions on the relative activity of extracellular maltase from *Bacillus licheniformis* KIBGE-IB4.

Metal Ions	1.0 mM		5.0 mM		10.0 mM	
	30.0 min	60.0 min	30.0 min	60.0 min	30.0 min	60.0 min Hr
Control	100.0	100.0	100.0	100.0	100.00	100.00
BaCl2	153.5	161.0	116.5	112.3	100.30	100.00
CoCl2	163.3	125.2	103.5	125.3	120.56	110.23
MnCl2	130.2	120.7	145.5	100.0	105.85	100.00
MgCl2	158.3	152.6	110.5	100.0	100.00	106.13
ZnCl2	150.0	70.3	79.5	60.2	50.56	50.31
CuCl2	143.4	90.5	70.0	50.1	45.60	40.05
CaCl2	116.6	110.8	126.3	110.5	108.90	110.50
CsCl2	141.6	120.0	147.4	110.9	100	100
NaCl	125.0	120.7	119.3	134.7	130.45	110.90
KCl	125.0	134.3	138.6	123.2	120.42	115.84
NiCl2	58.3	49.1	38.1	25.1	12.56	7.05
HgCl2	Nil	Nil	Nil	Nil	Nil	Nil
VOCl2	82. 6	50.1	51.9	46.1	40.45	39.90
AlCl3	75.6	40.1	32.0	30.4	14.34	Nil

no significant effect was noticed. It was also found that vanadyl sulfate (VOSO4) and aluminum chloride (AlCl3) at 1.0 mM concentration repressed activity and inhibited 17.32 % and 24.40 % activity respectively. Whereas, when the concentration of divalent cation increased to 10.0 mM, most of the metal complexes showed stabilizing effect. The current results suggested that the most of the metal ions have no adverse effect on activity of maltase and majority of them act as stabilizer.

The effect of various surfactants on the catalytic behavior of maltase was determined in order to investigate the ability of enzyme to perform hydrolysis in presence of these surfactants. The maximum tolerance to universal solvents like ethanol is a property

of interest in biocatalyst reactions to modify the compounds that are not soluble in water at elevated temperatures. The impact of organic solvents was checked at various concentrations and it was observed that among all the organic solvents, dimethyl sulfoxide (DMSO) and ethanol showed 50 % inhibition; whereas, all other solvents showed a slight inhibitory effect on enzyme (Table 3). The percent inhibition of maltase was increased with increased concentration of organic solvent. The loss of activity might be due to the structural alteration of some groups other than active site of the enzyme [36].

The molecular weight of the maltase was determined by using native-PAGE (Fig.3). The zymography was carried out by staining the

Table 3
Effects of different organic solvents on catalytic activity of maltase.

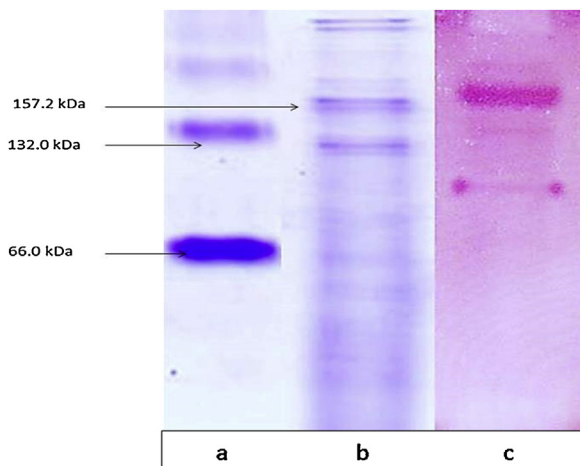| Solvents	Relative Activity (%)			
	1.0 mM	5.0 mM	10.0 mM	50.0 mM
Control	100.00	100.00	100.00	100.00
DMSO	50.00	48.50	34.65	15.23
Ethanol	50.00	43.87	30.50	24.91
Methanol	93.76	90.45	87.68	80.95
Isopropanol	90.55	90.78	82.33	80.70
Formaldehyde	93.67	92.56	89.35	87.85
Chloroform	80.56	80.60	78.90	76.58

Fig. 3. Native-PAGE and zymography of partially purified maltase. Lane A: BSA dimer and monomer protein marker; Lane B: dialyzed sample; Lane C: zymography of maltase from *B. licheniformis* KIBGE-IB4.

maltase containing gel with tetrazolium red dye. Molecular weight was measured by comparing the relative mobility of the enzyme with the molecular weight of marker used when stained with Coomassie brilliant blue R-250 and the molecular weight of maltase was assumed to be approximately 157.2 kDa. The results suggested that the maltase extracted from *B. licheniformis* KIBGE-IB4 displayed many protein bands as this is precipitated protein

4. Conclusion

Maltase is an industrially important catalytic protein that has broad utility profile to accelerate different biochemical reactions. It was concluded from the current study that the maltase from *B. licheniformis* KIBGE-IB4 has an excellent catalytic properties. In terms of stability, maltase showed higher tolerate at 40 °C in potassium phosphate buffer (0.1 M, pH-6.5). All tested divalent cations activated the maltase activity except Ni+2 and Hg+2. These characteristics indicates the potential applications of maltase for different commercial bioprocesses including food technology.

Declaration of Competing Interest

Authors declare no conflict of interest regarding publication of this manuscript.

Acknowledgment

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