INTRODUCTION

Microorganisms from nature have been found to contain a great variety of enzymes with tremendous variation in kinetic features, suitable for applications in diverse types of industries (Sidhu et al., 1997). Among various enzymes of industrial importance, α-amylase (EC 3.2.1.1) holds top position accounting for approximately 25% of the enzyme market (Sidhu et al., 1997). Most importantly, they find applications in starch processing, brewing and sugar manufacturing, desizing in textile industries and detergent manufacturing (Pandey et al., 2000). The α-amylases have been commercially produced applying bacteria belonging to the genus Bacillus such as B. amyloliquefaciens, B. stearothermophilus, B. subtilis and Bacillus licheniformis (Sivaramakrishnana et al., 2006).

Until now, there has been a wide interest in thermostable enzymes including thermostable α-amylases for obvious reasons (Arikan, 2007; Carvalho et al., 2008). It is recently, the cold-active enzymes have started arousing much industrial interest because of potential economic benefits such as less energy investment, less chance of contamination (Cavicchioli et al., 2002) and higher quality of products (Sahay et al., 2012; Sahay et al., 2013) as a result of cold processing that goes with these enzymes. Thus for certain industries such as those of food, detergent etc., the cold-active enzymes have started arousing much industrial interest because of potential economic benefits such as less energy investment, less chance of contamination (Cavicchioli et al., 2002) and higher quality of products (Sahay et al., 2012; Sahay et al., 2013) as a result of cold processing that goes with these enzymes. 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Partial purification

After 72h of growth, the culture was centrifuged at 5000 rpm to remove cells. The supernatant was saturated with ammonium sulphate to 40%, 50%, 60%, and 80% level and precipitated proteins were collected by centrifugation at 10000 rpm at 4ºC (REMI/CM12) for 10 min. Maximum activity was observed in 50% fraction, that was thus dialysed against 100 mM phosphate buffer (pH 7.0) at 4ºC, resuspended in the same buffer in minimum volume. The protein was further purified by DEAE chromatography as earlier (Roohi et al., 2013) and stored at -10ºC until used.

Enzyme assay

The reaction-mix containing 1 ml of 1% starch solution and 1 ml of properly diluted enzyme (kept at -10ºC) was incubated at 25ºC for 30 min. A 0.3ml of this solution was taken in another test tube, and equal volume of of 3.5-dinitrosalicylic acid reagent was added to it. The solution was then boiled for 5min, and then cooled down to room temperature. It was diluted with 2.7ml of distilled water and, absorbance was measured at 540 nm using UV-Vis spectrophotometer. One unit of amylase activity was defined as the amount of enzyme that released 1µM of reducing sugar equivalent to glucose per min under the assay condition (Miller, 1954). The experiments were performed in three sets of duplicate cultures, and the mean value of enzyme activity was determined.

Protein content

Protein concentration was determined by the method described earlier (Lowry et al., 1951) using bovine serum albumin as standard.

Effect of pH

The effect of pH on the enzyme activity was determined by varying the pH of the reaction mixtures using the following buffers (100 mM): sodium acetate (pH 3.0-6.0), sodium phosphate (pH 6.0-7.0), Tris-HCl (pH 8.0-9.0) and glycine-NaOH (pH 10-11) followed by assay of the enzyme. The pH stability was determined by pre-incubating the enzymes in different buffers for 1h. The residual activity was then assayed in 100 mM sodium phosphate buffer (pH 7.0).

Effect of temperature

The effect of temperature on the enzyme activity was evaluated by measuring the amylolytic activity at different temperatures (4ºC, 25ºC, 40ºC, 50ºC) in 100 mM glycine-NaOH (pH 11). The effect of temperature on stability of α-amylases was determined by pre-incubating it in 100 mM glycine-NaOH (pH 11.0) at 50ºC for 1h, 2h, 3h, 4h, 24h, 48h and 72h followed by measuring its residual activity. The enzymes were also tested for their stability at boiling temperature by boiling for 1min, 5 min, 10 min, 15 min, 20 min and 30 min followed by determining the residual activity.

Effect of metal ions and chelator

The effect of metal ions on amylases was determined by pre-incubation of the enzyme with various metal ions at a final concentration of 5 mM in glycine-NaOH buffer (pH 11.0), at 30ºC for 30 min followed by their assay for the residual activity. Likewise, enzymes were pre-incubated with EDTA or SDS to the final concentration of 5 mM and 1% (w/v) respectively at 30ºC for 30 min, to see their effect on amylolytic activity. To examine stability, the incubation period of the enzymes with above factors was extended to 60 min followed by their assay for the residual activity. The activity of the enzymes alone in 100 mM glycine-NaOH buffer (pH 11.0) was taken to be 100%.

Effect of detergent components

The effect of important detergent ingredients such as surfactants (SDS, Tween-80 and Triton X-100), and oxidizing (H₂O₂ and bleaching (NaClO₃) agents was studied by incubating enzyme (50 µl) with 950 µl of 1% of these reagents in 10 mM glycine-NaOH buffer (pH 11.0) for 1h and 2h at 40ºC (Chakraborthy et al., 2012) before assaying the enzyme as compared to the control without any additive.

Effect of laundry detergents

The detergent brands used were Surf Excel® (Hindustan Unilever Limited-Mumbai, India) and Tide® and Ariel® (Procter and Gamble Home Products Ltd). They were diluted in double distilled water to a final concentration of 7 mg ml⁻¹ to simulate washing conditions and heated at 100ºC for 15 minutes to inactivate the enzymes that could be part of their formulation (Carvalho et al., 2003). The detergents were added to the reaction mixture and the reaction was carried out under standard assay conditions. To determine the stability of CB13 amylase in the presence of the different detergents, an amylase concentration of 1 mg.ml⁻¹ was added in detergent solution and incubated at 50ºC for 12 h. Aliquots (0.5 ml) were taken at different time intervals and the residual activity determined at 20ºC and 4ºC and, compared with the control sample incubated at 50ºC without any detergent (Carvalho et al., 2008).

Taxonomic characterization of CB13

 Morphological and physiological characterizations of the most potential isolate, CB13 were carried out according to Barnett et al. (2000). All carbon sources except Arbutin, D-glucos-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-gluconate, D-gluconate, D-gluconate and D-galactonate and nitrogen sources have been tested for their utilization by the yeast isolate CB13. For molecular characterization, DNA isolation from yeast was performed as per the protocol given earlier (Harzu, 2004). PCR amplification of D1/D2 region of the large ribosomal subunit was carried out applying primers NL-1 (5′- GCATATCAATAGCCTGGAGGAAAAG-3′) and NL-4 (5′- GGTCCGTTTCAGACGCG-3′) and protocol given earlier (Kurtzman and Robnett, 1997). The purified ~600 bp PCR products were sequenced by automated DNA sequencer -350xL DNA analyzer by Applied Biosystems using Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequence analysis software version 5.2 from applied biosystems was applied to align sequence data and generation of dendrogram. The sequences obtained for upper and lower strands were manually aligned before performing the analysis. BLASTN was used to compare this sequence to the non-redundant NCBI database to find the most similar sequence and were sorted by the E score. A representative sequence of 10 most similar neighbours was retrieved and multiple alignment was performed applying CLUSTAL W2 with the default settings. The evolutionary history was inferred using the UPGMA method (Tamura et al., 2011) and evolutionary analyses were conducted in MEGA5.

RESULTS AND DISCUSSION

Isolation of alkali tolerant amylase producing yeasts

In the first round, yeasts were isolated from various rotten vegetables showing constitutive amylolytic activity. In the second round, the selected yeasts were tested for their growth performance on alkaline medium and consecutively five yeasts showing constitutive amylolytic activity at pH 9 were selected (Tab 1) for further study. Amylases have been reported from a number of yeasts (Wilson and Ingleed, 1982; Moranelli et al., 1982; DeMot and Verachtert, 1986; DeMot and Verachtert, 1987; Lefuji et al., 1996; Wanderley et al., 2004), there is hardly any report of their isolation from C. lasitaniae. Amylases are one of the most important industrial enzymes finding application in a variety of fields. Bacteria are the most important organisms serving the source of industrial grade amylases so far (Sajedi et al., 2005), though enzymes with unique set of biochemical characteristics fit for specific applications have been reported from other classes of microbes (Sivaramakrishnan et al., 2006). As to the cold-active amylases, hardly any class of microbes other than bacteria of Antarctica origin has been explored (D’Amico et al., 2003). Therefore, cold-active amylases from these bacteria have become the model for biochemical study (D’Amico et al., 2003) and, contemplating future applications. Likewise, alkali amylases have been isolated from various sources earlier (Horikoshi, 1999). We are reporting cold-active amylolytic activity with utterly novel features from a yeast-isolate, C. lasitaniae CB13 isolated from rotten cabbage.

Enzyme production

During growth on agar plates containing starch as substrate, selected yeast isolates formed large starch digesting halos around their colonies as were evident from staining with iodine solution. This was an indication of the presence of amylolytic activity. The degradation of starch was accompanied by cell growth and amylase secretion in the production medium. The extracellular amylolytic activity increased during cell growth and reached maximum values at 96 h of incubation, which continued till 120 h (data not shown). The optimum enzyme activity at 96 h as shown by CF2, SP1, SP3, CB13 and PTC were 41.6, 13.92, 41.6, 46.7 and 11.1 U/ml for 0.1 % substrate concentration respectively (Tab 1).
Table 1 Maximum activity, optimum pH and optimum temperature of amylases isolated from selected yeast isolates

<table>
<thead>
<tr>
<th>Strains</th>
<th>Source</th>
<th>Maximum activity (U/ml/min)</th>
<th>Optimum pH</th>
<th>Optimum temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF2</td>
<td>Brassica oleracea</td>
<td>41.6</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>SP1</td>
<td>Spinacia oleracea</td>
<td>13.92</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>SP3</td>
<td>Spinacia oleracea</td>
<td>41.6</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>CB13</td>
<td>Brassica oleracea var.</td>
<td>46.17</td>
<td>11</td>
<td>40</td>
</tr>
<tr>
<td>PTC</td>
<td>Allium cepa</td>
<td>11.11</td>
<td>11</td>
<td>40</td>
</tr>
</tbody>
</table>

Preliminary characterization of enzyme from selected yeast-isolates

In case of most strains, 0.1% substrate concentration was found to be optimum. Only in case of CB13, a 0.2% substrate concentration was found to be optimum (data not shown).

The amylases from selected isolates were assayed at various pHs (Fig 1a). All the isolates seemed to yield alkaline amylases as the activity was found maximum at pH 11. While CB13 seemed to be most pH-sturdy isolate since its activity profile fell to only 55% while that of CF2 to 32% (Fig 1a). Overall, the amylases from all isolates showed one major peak activity at pH 11 and a minor one at near pH 6.8. The activity of enzyme (amylases) was also assayed at 4°C, 25°C and 40°C. The amylases from all the isolates showed activity at all the temperatures though optimum temperature differ slightly, being 40°C for all of them except CF2 (Fig 1b). The activity from all the isolates was found to continue down to 4°C. The percentage of amylases activity at 4°C varied from 84% (SP3) to 46 (SP1 and CB13) which is noteworthy.

Further characterization of amylase from CB13

The enzymes could resist boiling for 1 min, during which a loss of only 10% of its activity was recorded. The enzymes retained 20% residual activity after 30 min of boiling (Fig 2).

Figure 2 Effect of temperature (boiling temperature) on CB13 amylolytic activity

Ca²⁺ was found to have almost no effect on the enzymes immediately; though a little improvement of activity (8%) was observed after 1h of incubation with it. Among inhibitors, Cu²⁺ had drastic effect reducing activity to 16% whilst Fe³⁺ reduced the activity marginally by 10%. Among activators, Mn²⁺ was found to be the most effective one, followed by Co²⁺ and Mg²⁺ in that order. None of the metal ions tested was found to affect stability of the enzyme (Fig 3).

Figure 3 Effect of metal ions, chelator, salt and SDS on CB13 amylolytic activity

The activity of CB13 enzymes was found to be stimulated by salt, EDTA and SDS. SDS stimulated the activity by 80% during 1h of its incubation with the enzymes. The enzymes showed 100% to more than 80% of residual activity after 2h of incubation with selected detergent ingredients (Fig 4a). The enzymes exhibited retention of about 80% of their maximum activity till 6h of incubation in presence of various detergents (Fig 4b) as compared to control; a restoration of the activity was also seen in next 6h (Fig 4b).
The yeast isolate CB13 was characterized morphologically and physiologically and assigned to the genus *Clavispora lusitaniae*. The colony was white in colour and butyrous in texture. Formations of pseudohyphae and budding cells (both unipolar and bipolar) were found. There was formation of 1–4 (typically four) ascospores per cell, each ascospore was smooth (Fig. b). The physiological data, when compared to CBS database with respect to *C. lusitaniae*, was found to be almost similar except in the utilization of starch as carbon source and Nitrate, Creatine, Creatinine and Tryptophan as nitrogen source. As against the CBS strains of *C. lusitaniae*, CB13 could utilize these metabolites as sole carbon or nitrogen source as the case may be.

**Figure 6** Phylogenetic relationship of CB13 with 10 closely related isolates retrieved from Genbank on the basis of D1/D2 sequences.

Earlier, *C. lusitaniae* has been reported to be associated with diseased lesions (Merz et al., 1990; Gargeya et al., 1992). Since the amylolytic activity of this strain is attractive, expression of this activity in an appropriate industrial microbe may be a safer way to exploit it (Steven and Pretorius, 1995). Physiological deviation in carbon and nitrogen utilization profile (Table 2) exhibited by CB13 as compared to those of other CBS strains of *C. lusitaniae* indicates the genetic flexibility of this taxon making it an adaptable organism. Moreover, this is the first report of stable, alkaline and cold active amylolytic activity from yeast with potential application in detergent.

**Reproducibility of results**

All the experiments were carried out in triplicates and five times. Unless otherwise indicated, all values are average values calculated from three independently derived sets of data.

**CONCLUSION**

The yeast-isolates showing alkaline amylolytic activity were isolated from rotten vegetables. From among them, CB13 amylolytic activity was selected on the
basis of pH and temperature robustness for further characterization. The enzymes of CB13 were thermostable, Ca²⁺ independent, non-metalloenzyme showing compatibility with surfactants, bleaches, oxidizing agents and local powder detergents. These features suggested the CB13 enzymes to be suitable candidate for detergent preparations. The yeast-isolate CB13 was identified as C. lusitaniae on the basis of morphological, physiological and molecular characteristics.

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REFERENCES


