JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

DETERGENT COMPATIBLE COLD-ACTIVE ALKALINE AMYLASES FROM *CLAVISPORA LUSITANIAE* CB13

*Kamlesh Ranjan, Mansoor Ahmad Lone, Sanjay Sahay**

Address(es):
Government Science & Commerce College, Benazir, Bhopal (MP) India.

*Corresponding author: ss000@rediffmail.com

doi: 10.15414/jmbfs.2016.5.4.306-310

ARTICLE INFO

Received 14. 5. 2014
Revised 22. 6. 2015
Accepted 22. 10. 2015
Published 1. 2. 2016

Regular article

OPEN ACCESS

ABSTRACT

After two stages of screening, five environment isolates of yeasts showing amylolytic activity at alkaline pH and residual activity at cold temperatures were isolated from rotten vegetables. Based on pH and temperature robustness, amylases from CB13 were selected for further characterization. The enzymes from this yeast-isolate showed optimum activity at pH 11 and temperature 40°C, 42 % residual activity at 4°C, no dependence on Ca^{2+} for activity and stability, resistance to EDTA and SDS, and amplification in activity in presence of Mn^{2+} and Co^{2+}. The amylolytic activity was thermostable showing retention of 50% of optimum activity after boiling for 30 min. The enzymes retained nearly 80% of activity after exposing to various detergent components, and also to commercially available laundry detergents for 2h. The morphological, physiological and molecular characterization of the isolate CB13 led to its identification as *Clavispora lusitaniae*. This is the first report on the screening of detergent compatible α-amylase with residual cold-activity from yeast isolated from rotten vegetables.

Keywords: *Clavispora lusitaniae*, Alkaline α-amylases, Detergent compatible α-amylases, Cold-active α-amylase

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 are now considered to be highly desirable (**Nakagawa *et al*., 2002; Roohi *et al*., 2013; Sahay *et al*., 2013**). One of the essential characteristics of cold-active enzymes viz., thermal instability is however a major hindrance in certain application such as detergent. Amylases are one of the highly used enzymes in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes (**Hmidet *et al*., 2009**).
The enzymes, amylases have many areas of applications; each application requires a special set of properties with respect to thermostability, pH profile, pH stability, Ca^{+2}-independency etc. For example, amylases used in starch industry must be active and stable at low pH but those used in detergent industry must be active and stable at high pH, Ca^{2+}-independent and resistant to detergent components such as certain surfactants and oxidizing, chelating and bleaching agents (**Carvalho *et al*., 2008; Chakraborty *et al*., 2012**). It is, therefore, important to explore various sources to isolate such microbe as producing amylases with desirable features for specific application. The psychrotrophic microbes so far yielded very unstable amylases with hardly desirable features for application to detergent formulation (**Roohi *et al*., 2013**). The present paper describes isolation and partial characterization of cold active alkaline α-amylases from a mesophilic natural yeast for application in detergent industry.

MATERIALS AND METHODS

Site, sampling and source materials

Various rotten vegetables were collected from the local market of Bhopal (34.5°–36° South latitude and 70°–66.5°Western longitude) and carried to laboratory aseptically. For inoculums preparation, vegetables after cutting into pieces of about 1mm^2 with sterile knife were placed in flasks containing 10 ml of sterile saline water (0.1%) to a final concentration of 0.1 gm ml^{-1}. The sample was shaken at165 rpm for 1 h at room temperature. Aliquot of 0.1 ml size was spread on the surface of PDA (potato, glucose, agar) medium supplemented with chloramphenicol in the concentration of 1mg ml^{-1}. The plates were incubated at 25°C for 72 h.

Screening of extracellular amylase secreting isolates

Medium used was a modified minimal synthetic medium (MM) containing (g l^{-1}); $(NH_4)_2SO_4$-5, KH_2PO_4-1, NaCl-0.1, $MgSO_4$-0.5, $CaCl_2$-0.01, starch-0.1, Agar-15, and pH adjusted to 7.0 (**Sahay *et al*., 2013**). The MM contained starch as sole carbon source to screen yeast isolates exhibiting constitutive amylolytic activity. The plates were incubated at 25°C for 48h. Amylase production was detected after flooding the plates with lugol's iodine (**Arikan, 2008**).

Second screening

In the second round of selection, the main parameters used were the rate of colony-growth and ability to grow at higher pH. The medium used was MM with pH adjusted to 7.0 and 9.0 with 0.1 N NaOH. Since agar is not gelled at higher pHs properly, psyllium-gelled medium was also used (**Sahay, 1999**).

Enzyme production

One ml of log phase culture (density 10^6 l^{-1}) from yeast growth medium (0.67% yeast nitrogen base without amino acids and 2% glucose) was transferred to 250-ml conical flask containing 50 ml of the yeast production medium (0.67% YNB and 2% starch). The growth was carried out at 25°C in a refrigerated shaking incubator (REMI/CIS 4) at 150 rpm, and was monitored by measuring OD_{600} of growth medium. Aliquots of 5 ml sizes were withdrawn at 12h intervals, cells were spun down at 5000 rpm for 10 min and the supernatant was used to assay α-amylases.

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 4°C (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 4°C, 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 -10°C until used.

Enzyme assay

The reaction-mix containing 1 ml of 1% starch solution and 1 ml of properly diluted enzyme (kept at -10°C) was incubated at 25°C for 30 min. A 0.3ml aliquot 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 (4°C, 25°C, 40°C, 50°C) 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 50°C 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 30°C 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 30°C 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 glycing-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 (H_2O_2) and bleaching ($NaClO_3$) 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 40°C (**Chakraborty *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*., 2008**). 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 4°C 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 Barnet et al. (2000). All carbon sources except Arbutin, D-glucono-1,5-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, D-gluconate, D-glucuronate, D-glucarate 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'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') and protocol given earlier (**Kurtzman and Robnett, 1997**). The purified ~600 bp PCR products were sequenced by automated DNA sequencer -3037xl DNA analyzer from 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 alkalitolerant 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 Ingledew, 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. lusitaniae*. 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, alkaliphiles 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.lusitaniae* 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 11.11 U/ml for 0.1 % substrate concentration respectively (Tab 1).

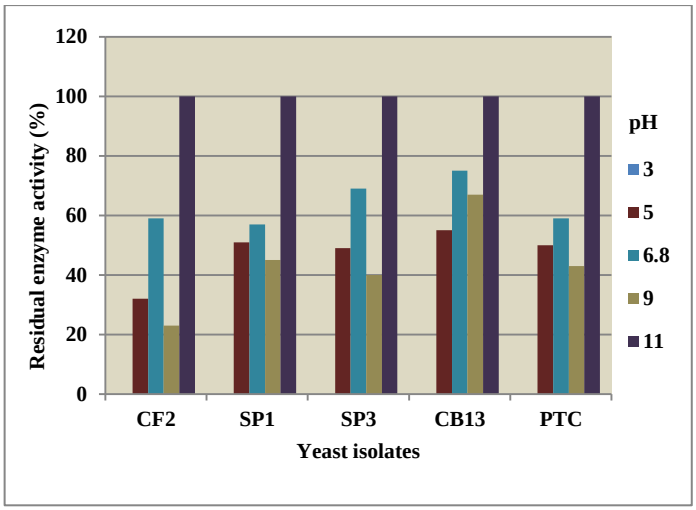
Table 1 Maximum activity, optimum pH and optimum temperature of amylases isolated from selected yeast isolates

Strains	Source	Maximum activity (U/ml/min)	Optimum pH	Optimum temperature (°C)
CF2	*Brassica oleracea*	41.6	11	25
SP1	*Spinacia oleracea*	13.92	11	25
SP3	*Spinacia oleracea*	41.6	11	25
CB13	*Brassica oleracea* var. capitata	46.17	11	40
PTC	*Allium cepa*	11.11	11	40

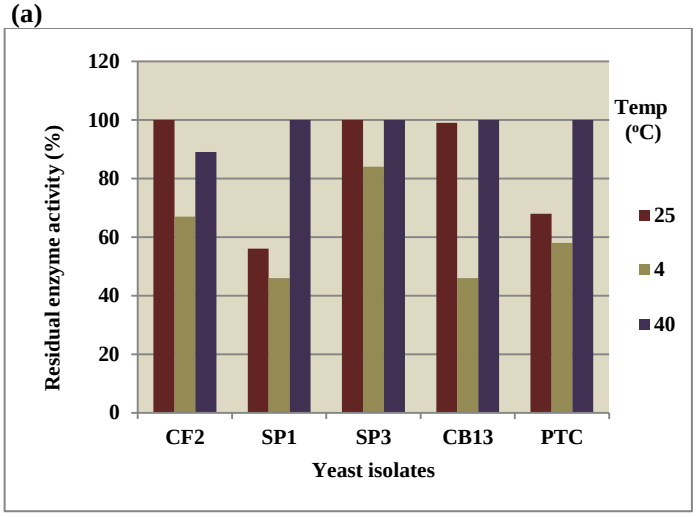
Preliminary characterization of enzyme from selected yeast-isolates

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 pH11. 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 and 50°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.



(a)



(b)

Figure 1 Effect of pH (a) and temperature (b) on the extracellular amylolytic activity in the partially purified extract from five selected yeast-isolates.

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).

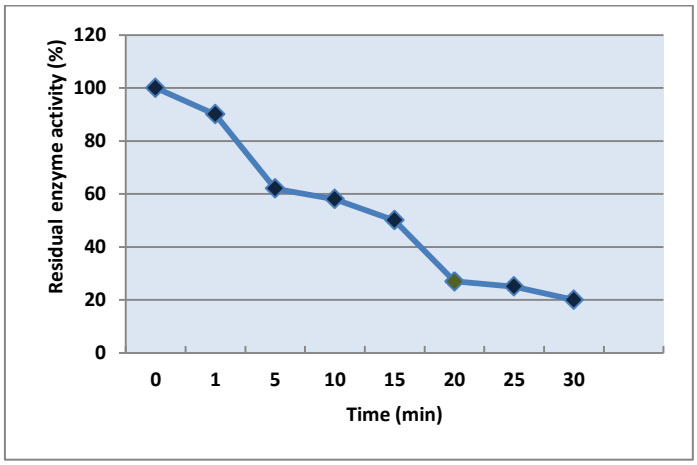


Figure 2 Effect of temperature (boiling temperature) on CB13 amylolytic activity

Ca^{2+} 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, Cu^{2+} had drastic effect reducing activity to 16% whilst Fe^{3+} reduced the activity marginally by 10%. Among activators, Mn^{2+} was found to be the most effective one, followed by Co^{2+} and Mg^{2+} in that order. None of the metal ions tested was found to affect stability of the enzyme (Fig 3).

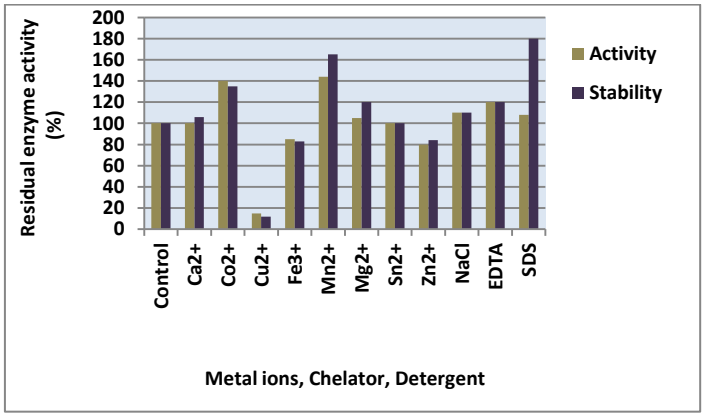
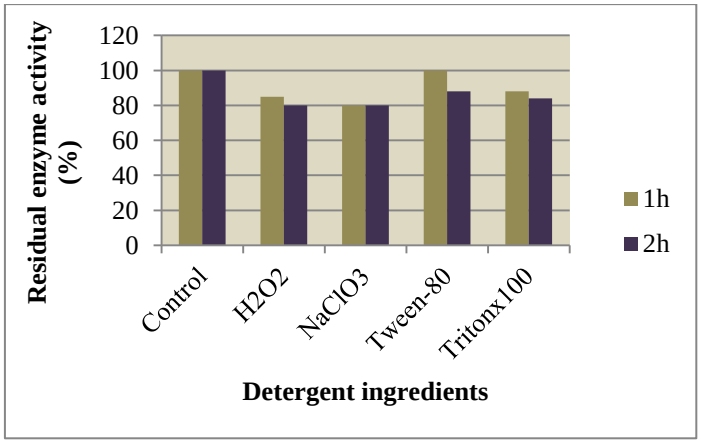
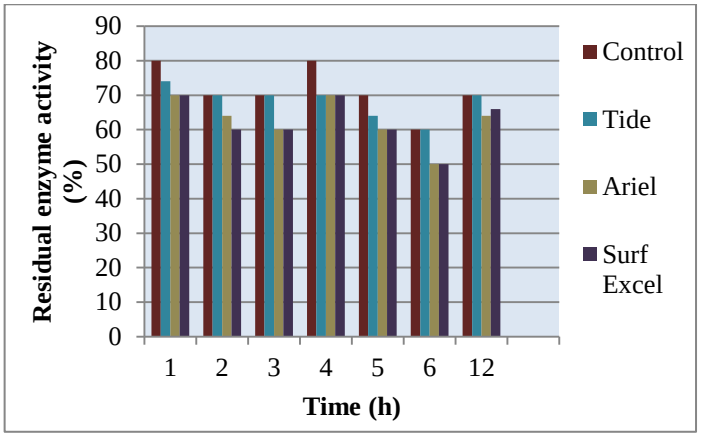


Figure 3 Effect of metal ions, chelator, salt and SDS on CB13 amylolytic activity

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).



(a)



(b)

Figure 4 Effect of (a) detergent ingredients (components) and (b) laundry detergents on CB13 amylolytic activity.

The CB13 amylolytic activity showed optimum activity at 40°C at pH 11.0 which is not in agreement with the data for the amylases from *S. alluvius* ATCC 26074 (**DeMot and Verachtert, 1986**), *S. alluvius* UCD 54-83 (**DeMot and Verachtert, 1987**) *L. kononenkoae*, *C. antarctica* CBS 6678 (**DeMot and Verachtert, 1987**) and *C. flavus* (**Wanderley *et al*., 2004**). The yeast enzymes retained 45% and 98% of their maximum activity at 4°C and 25°C, a feature that may enable their application in various seasons and over larger geographical areas.

The enzyme is comparatively thermostable at lower temperatures, and also during boiling. It retains 20% residual activity after boiling for 30 min, a feature which is remarkable not reported so far. Amylases have earlier been reported, but they required Ca^{2+} for their stability at higher temperature e.g., *Bacillus* sp. I-3, *Bacillus* sp. ANT-6, *B. subtilis*, *Bacillus clausii* BT- 21 and *Bacillus licheniformis* (**Carvalho *et al*., 2008**). This is thus the first report of yeast amylases showing thermostability at par with the bacterial's one, but independent of the presence of Ca^{2+} for activity or stability.

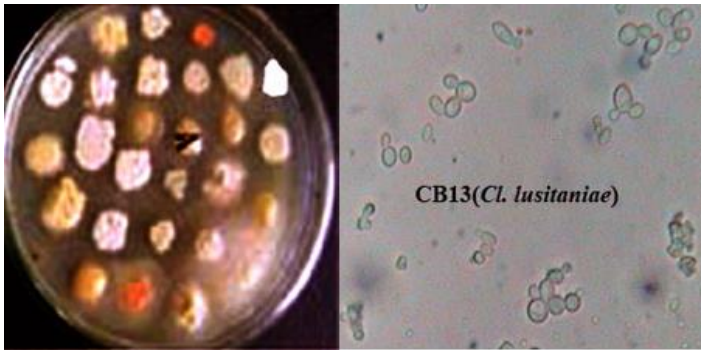
The optimal pH of the enzymes is 11.0 which is again a deviation from general acidic yeasts-amylases with optimum pH usually in the range of 4.0 and 6.0 (**Kelly *et al*., 1985; Lefuji *et al*, 1996; Wanderley *et al*., 2004**). Moreover, more than 50% of the activity of the enzymes was retained between pH 5.0 to 11.0 which is something unusual, hardly reported so far in respect of amylolytic activity from any organism. This unusual pH sturdiness is an attractive feature and forms the basis for their various industrial applications. Alkalitolerant microbes have been evaluated as important sources of various bioactive substances (**Horikoshi, 1999**), hardly a few studies have been made with respect to alkalitolerant yeasts (**Duckworth *et al*., 1996; Lisichkina *et al*., 2003**).

As opposed to earlier reports, the enzymes were inhibited by only few metal ions studied. Among inhibitors of the enzymes, Cu^{2+} and Fe^{3+} ions are general inhibitors of amylases as reported earlier (**Kelly *et al*., 1985; Aguilar *et al*., 2000; Wanderley *et al*., 2004**).Likewise, Mn^{2+} and Co^{2+} were found to be very effective activators for these enzymes as for bacterial amylases (**Bernharsdotter *et al*., 2005**). None of the metal ions tested was found to reduce the stability, rather one of them (Mn^{+2}) was found to enhance the activity during incubation with it. The enzymes were found to be moderately stimulated and stabilized by NaCl. The facts that the enzymes activity was only moderately stimulated but not stabilized by Ca^{+2} and that the activity was not affected by EDTA suggest that the enzymes are not metalloenzyme. In most of the cases, amylases have been found to be Ca^{+2} dependent metalloenzyme (**Syed *et al*., 2009**), though a few Ca^{2+} independent amylases have also been reported (**Alva *et al*., 2007**). The Ca^{2+} independent amylases are considered to be suitable for application in detergents.

The amylolytic activity was most surprisingly enhanced by SDS indicating that hydrogen bonds may not play a key role in maintaining enzyme activity (**Wang *et al*., 2005**). The enzymes were not inhibited by NaCl indicating that it is dependent on Cl^{-1}, a feature it shares with the Antarctica bacterium *P. haloplanctis* amylase and others (**Numao *et al*., 2002**). The unique characters of the enzymes were also manifested during incubation with various commercial detergents showing retention of about 80% of its maximum activity till 1h. The enzymes seem to be affected by the chemical environment of detergent to a little extent but yet, it showed much higher resistance to detergent as compared to earlier reported amylases (**Carvalho *et al*., 2008**). The biochemical properties of CB13 amylolytic activity, therefore, differ in a great deal from all the previously reported amylases (**Sivaramakrishnan *et al*., 2006**). It appears to be a cold-active nonmetallo-enzyme with a number of novel features. Preliminary biochemical characteristics suggest that these activities may find application in laundary detergent and textile industry. Since, amylases are one of the highly used enzymes in the formulation of enzymatic detergent (**Hmidet *et al*., 2009**), the enzymes may be commercially very important.

Taxonomic characterization of CB13

The yeast isolate CB13 was characterized morphologically and physiologically and assigned to the genus *Clavispora lusitaniae*. The colony was white in colour and butyrus 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.



(a) **(b)**

Figure 5 (a) Colonies of selected yeast-isolates including that of CB13 and (b) cells of CB13 with ascospores.

For molecular identification, the D1/D2 regions of 26s rDNA of CB13 was sequenced and the sequence was compared with those available in the NCBI database. The phylogenetic tree based on this sequence of CB13 along with those from ten the most closely related strains obtained from NCBI database showed 100 % similarity to type and many of other strains of *Clavispora lusitaniae* (Fig.6), therefore, the isolate was so named. The nucleotide sequence has been deposited in the GenBank database under Accession number JNO91166

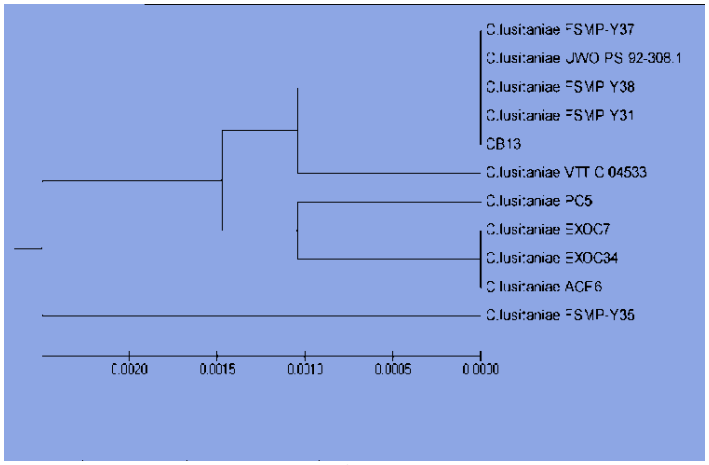


Figure 6 Phylogenetic relationship of CB13 with 10 closely related isolates retrieved from Genbank on the basis of D1/D2 sequences.

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, Ca^{2+} 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.

Acknowledgement: The research grant from University Grants Commission (India) to the corresponding author is herewith gratefully acknowledged.

REFERENCES

AGUILAR, G., MORLON-GUYOT, J., TREJO-AGUILAR, B., GUYOT, J.P. 2000. Purification and characterization of an extracellular α-amylase produced by *Lactobacillus manihotivorans* LMG 18010T, an amylolytic lactic acid bacterium. *Enzyme and Microbial Technology*, 27, 406–413. http://dx.doi.org/10.1016/S0141-0229(00)00230-1

ARIKAN, B. 2008. Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic Bacillus sp. isolate A3-15. *Bioresource Technology*, 99 (8), 3071-3076 http://dx.doi.org/10.1016/j.biortech.2007.06.019

ALVA, S., ANUPAMA, J., SAVLA, J., CHIU, Y.Y., VYSHALI, P., SHRUTI, M., et al. 2007. Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. *African Journal of Biotechnology*, 6, 576-581.

BARNET, J.A., PAYNE, R.W., YARROW, D. 2000. Yeast, characterization and identification, 3rd edition. Cambridge, U.K. : University Press, ISBN: 13: 978-0521573962.

BERNHARSDOTTER, E.C.M.J., NG, J.D., GARRIOTT, O.K., PUSEY, M.L. 2005. Enzymic properties of an alkaline chelator-resistant α-amylase from an alkaliphilic *Bacillus* sp. isolate L1711. *Process Biochemistry*, 40, 2401–2408. http://dx.doi.org/10.1016/j.procbio.2004.09.016

CARVALHO, R.V.D.E., CÔRREA, T.L.R., DA SILVA, J.C.M., MANSUR, L.R.C. DE OLIVEIRA, MARTINS, M.L.L. 2008. Properties of an amylase from thermophilic *Bacillus* sp. *Brazilian Journal of Microbiology*, 39, 102-107.

CAVICCHIOLI, R., SIDDIQUI, K.S., ANDREWS, D., SOWERS, K.R. 2002. Low temperature extremophiles and their applications. *Current Opinion in Biotechnology*, 13, 253-261. http://dx.doi.org/10.1016/S0958-1669(02)00317-8

CHAKRABORTY, C., RAUT, G., KHOPADE, A., MAHADIK, K., KOKARE, C. 2012. Study on calcium ion independent amylase from haloalkaliphilic merine Streptomyces strain A3. *Indian Journal of Biotechnology*, 11, 427-437.

D'AMICO, S., MARX, JEAN-CLAUDE, GERDAY, C., FELLER, G. 2003. Activity-Stability Relationships in Extremophilic Enzymes. *Journal of Biological Chemistry*, 278 (10), 7891–7896. http://dx.doi.org/ 10.1074/jbc.M212508200

DE MOT, R., VERACHTERT, H. 1986. Secretion of α-amylase and multiple forms of glucoamylase by the yeast *Trichosporon pullulans*. *Canadian Journal of Microbiology*, 32, 47-51. http://dx.doi.org/ 10.1139/m

DE MOT, R., VERACHTERT, H. 1987. Purification and characterization of extracellular α-amylase and glucoamilase from the yeast *Candida antarctica* CBS 6678. *European Journal of Biochemistry*, 164, 643-654. http://dx.doi.org/ 10.1111/j.1432-1033.1987.tb11175.x.

DUCKWORTH, A.W., GRANT, W.D., JONES, B.E., VANSTEENBERGEN, R. 1996. Phylogenetic diversity of soda lake alkaliphiles. *FEMS Microbiological Ecology*, 19, 181-191. http://dx.doi.org/ 10.1111/j.1574-6941; http://dx.doi.org/ 1996.tb00211.x

GARGEYA, I.B., PRUITT, W.R., SIMMONS, R.B., MEYER, S.A., AHEARN, D.G. 1990. Occurrence of *Clavispora lusitaniae*, the teleomorph of *Candida lusitaniae*. *Journal of Clinical Microbiolgy*, 28, 2224-2227.

GUPTA, R., GIGRAS, P., MOHAPATRA, H., GOSWAMI, V.K., CHAUHAN, B. 2003. Microbial α-amylase: a biotechnologicalperspective. *Process Biochemistry*, 38, 1599-1616. http://dx.doi.org/ 10.1016/S0032-9592 (03)00053-0

HADFIELD, T.L., SMITH, M.B., WINN, R.E., RINALDI, M.G., GUERRA, C. 1987. Mycoses caused by *Candida lusitaniae*. *Review of Infectious Diseases*, 9:1006-1012.

HARJU, S. 2004. Rapid isolation of yeast genomic DNA: Bust n' Grab. *BMC Biotechnology*, 4, 8. http://dx.doi.org/10.1186/1472-6750-4-8

HORIKOSHI, K. 1999. Alkaliphiles: Some applications of their products for biotechnology. *Microbiology and Molecular Biology Review*, 63(4), 735-750.

KELLY, C.T., MORIARTY, M.E., FOGARTY, W.M. 1985. Thermostable extracellular α-amylase and α-glucosidase of *Lipomyces starkeyi*. *Applied Microbiology and Biotechnology*, 22, 352-358.

KURTZMAN, C.P., ROBNETT, C.J. 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26s) ribosomal DNA gene. *Journal of Clinical Microbiology*, 35, 1216–1223.

LEFUJI, H., CHINO, M., KATO, M., IIMURA, Y. 1996. Raw-starch digesting and thermostable α -amylase from the yeast *Rhodotorula* sp. S-2: purification, characterization, cloning and sequencing. *Biochemical Journal*, 318, 989-996.

LISICHKINA, G.A., BAB'EVA, I.P., SOROKIN, D.Y. 2003. Alkalitolerant yeasts from natural biotopes, *Microbiology*, 72, 695-698..

LOWRY, O.H., ROSEBROUGH, N., FARR, A., RANDALL, R. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275.

MERZ, W.G., KHAZAN, U., JABRA-RIZK, M.A., WU, LUNG-CHI, OSTERH, G.J. 1992. Strain Delineation and Epidemiology of *Candida* (*Clavispora*) *lusitaniae Journal of Clinical Microbiology*, 30, 449-454.

MILLER, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426-428. http://dx.doi.org/10.1021/ac60147a030

MORANELLI, F., YAGUCHI, M., CALLEJA, B., NASIM, A. 1982. Purification and characterization of the extracellular α-amylase activity of the yeast *Schwanniomyces alluvius*. *Biochemistry Cell Biology*, 65, 899-907. http://dx.doi.org/ 10.1139/o87-116

NUMAO, S., MAURUS, R., SIDHU, G., WANG, Y., OVERAL, C.M., BRAYER, G.D., WITHERS, S.G. 2002. Probing the Role of the Chloride Ion in the Mechanism of Human Pancreatic α-amylase. *Biochemistry*, 41, 215-225. http://dx.doi.org/10.1021/bi0115636

PANDEY, A., NIGAM, P., SOCCOL, C.R., SOCCOL, V.T., SING, D., MOHAN, R. 2000. Advences in microbial amylases. *Biotechnology and Applied Biochemistry*, 31, 135–152.

ROOHI, KOODUS, M., SAIMA 2013. Cold-active detergent-stable extracellular α-amylase from Bacillus cereus GA6: Biochemical characteristics and its perspectives in laundry detergent formulation. *Journal of Biochemical Technology*, 4(4), 636-644.

SAJEDI, R.H., NADERI-MANESH, H., KHAJEH, K., AHMADVAND, R., RANJBAR, B., ASOODEH, A., MORADIAN, F. 2005. A Ca-independent α-amylase that is active and stable at low pH from the *Bacillus* sp. KR-8104. *Enzyme and Microbial Technology*, 36, 666-671 http://dx.doi.org/10.1016/j.enzmictec.2004.11.003

SAHAY, S. 1999. The use of psyllium as alternative gelling agent for microbial culture media. *World Journal of Microbiology Biotechnology*, 15, 733-735 http://dx.doi.org/10.1023/A:1008954128637

SINGH, P., HAMID, B., LONE, M.A., RANJAN, K., KHAN, A., CHAURSE, V.K., SAHAY, S. 2012. Evaluation of pectinase activity from the psychrophilic fungal strain *Truncatella angustata* BPF5 for use in wine industry. *Journal of Endocytobiosis and Cell Research*, 22, 57-61.

SAHAY, S., HAMID, B., SINGH, P., RANJAN, K., CHAUHAN, D., RANA, R.S., CHAURSE, V.K. 2013. Evaluation of pectinolytic activities for oenological uses from psychrotrophic yeasts, *Letters in Applied Microbiology*, 57, 115-121. http://dx.doi.org/10.1111/lam.12081

SIDHU, G.S., SHARMA, P., CHAKRABART, T. et al. 1997. Strain improvement for the production of a thermostable α-amylase. *Enzyme and Microbial Technology*, 21, 525-530. http://dx.doi.org/10.1016/S0141-0229(97)00055-0.

SIVARAMAKRISHNANA, S., GANGADHARAN, D., NAMPOOTHIRI, K.M., SOCCOL, C.R., PANDEY, A. 2006. α-Amylases from Microbial Sources. *Food Technology and Biotechnology*, 44 (2), 173–184.

SYED,D.G., AGASAR, D., PANDEY, A. 2009. Production and partial purification of α–amylase from a novel isolate *Streptomyces gulbargensis*. *Journal of Industrial Microbiology and Biotechnology*, 36, 189-194 http://dx.doi.org/10.1007/s10295-008-0484-9.

STEYEN, A.J.C., PRETORIUS, I.S. 1995. Characterization of a novel α-Amylase from *Lipomyces kononenkoae* and expression of its gene (LKA1) in *Saccharomyces cerevisiae Current Genetics*, 28, 526-533. http://dx.doi.org/ 10.1007/BF00518165

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M., KUMAR, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, 28, 2731 –2739. http://dx.doi.org/ 10.1093/molbev/msr121

WANDERLEY, K.J., TORRES, F.A.G., MORAES, L.M.P., ULHOA, C.J. 2004. Biochemical characterization of α-amylase from the yeast *Cryptococcus flavus*. *FEMS Microbiology Letters*, 231, 165-169. http://dx.doi.org/10.1016/S0378-1097(03)00955-8

WANG, Q.F., MIAO, J.L., HOU, Y.H., DING, Y., WANG, G.D., LI, G.Y. 2005. Purification and characterization of an extracellular cold-active serine protease from the psychrophilic bacterim *Colwellia* sp. NJ341. *Biotechnology Letters*, 27, 1195-1198. http://dx.doi.org/10.1007/s10529-005-0016-x.

WILSON, J.J., INGLEDEW, W.M. 1982. Isolation and characterization of *Schwanniomyces alluvius* amylolytic enzymes. *Applied and Environmental Microbiology*, 44, 301-307.