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Research Paper

COLD-ACTIVE α-AMYLASE FROM PSYCHROPHILIC AND PSYCHROTOLERANT YEAST

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Abstract

The two yeast strains were isolated and identified as *Cystofilo basidium capitatum* SPY11 (psychrophilic) and *Rhodotorulamucilaginosa*PT1 (psychrotolerant). Both yeast isolates displayed momentous potential of secretingα-amylase under optimal conditions. The maximal specific α-amylase activity was found in*Rhodotorulamucilaginosa*PT1 2.17 U/ml/min at 25^{0}C and dramatically decrease in α-amylolytic activity were observed at 4^{0}C and 40^{0}C which is consistent with the feature of the cold-adaptive enzymes. Where as psychrophilic yeast strain *Cystofilo basidium capitatum* SPY11 exhibited maximal specific α-amylase activity 1.63 U/ml/min at 4^{0}C reflecting the nature of cold-active enzymes. *Cystofilo basidium capitatum* SPY11 and *Rhodotorulamucilaginosa*PT1 secreted neutral α-amylase with 100% activity at pH 7.0. Although *Cystofilo basidium capitatum* SPY11 showed second peak with 58% of α-amylase activity at pH 9.0.The α-amylase secreted by yeast isolate *Cystofilo basidium capitatum* SPY11 arecold-active in nature whereas *Rhodotorulamucilaginosa*PT1 α-amylase possess cold-adaptive features both having optimal pH of 7.0, this is considered asvirtuous advantage for textile, detergent, food as well as for biotechnological industry.

Key words: Psychrophilic yeasts, α-amylase activity,potential application.

INTRODUCTION

Amylases are enzymes that hydrolyze starch molecule to give diverse products including dextrin and progressively smaller polymers made up of glucose units [1, 2]. Amylases have been estimated to comprise approximately 30% of the world's enzyme production [3].Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents Though amylases originates from different sources (plants, animals and microorganisms), the microbial amylases are the most produced and used in industry, due to their productivity and thermo stability [4].

Yeasts are unicellular fungi with ubiquitous distribution in many ecosystems [5].Many yeast species have been found to produce amylases [6,7]including *Lipomyceskononenkoae*[8], *Schwanniomycesalluvius*[9,10], *Trichosporonpullulans*[11], *Candida antarctica*[12]and *Cryptococcus flavus*[13].Enzymes produced by microorganisms existing in cold environments display higher catalytic efficiency at low temperatures and greater thermosensitivity than their mesophilic counterparts [14].The psychrophilic yeasts and their cold-active enzymes have potential applications in biotechnology and other field[15]. Psychrophilic and psychrotolerant microorganisms and their unique cold shock and cold-acclimation proteins and enzymes (e.g.,

proteases, amylases, lipases and cellulases) having a host of biotechnology applications [16]. Psychrophilic yeasts play an essential role in nutrient cycling and biomass production processes in cold ecosystems[17]. Processes catalyzed by cold-active enzymes have two advantages: they have potential to economise the processes by saving energy [18,19], and they protect the processes from contamination [20]. Cold-adaptive α-amylase from Antarctica bacterium *Pseudoalteromonasplanktis*is best characterized cold-active enzyme that has been serving as model to study enzyme catalysis at low temperature [21,22]. There are only few reports on cold-active amylases from other bacteria [23], Actinomycetes[24], earthworm [25].
Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry[3,26].With the emergence of new frontiers in biotechnology, the scope of amylases has extended beyond their conventional applications in starch saccharification and in the textile, food, brewing and distilling industries to many other fields, such as clinical, medicinal and analytical chemistry [1].In this paper I report on two yeast isolates secreting α-amylases that are able to hydrolyze starch at low temperature, reflecting novel applications in biotechnological industries.

MATERIALS AND METHODS

Site, sampling and yeast isolation

Soil from a depth of 10cm from ten locations of Baramullah (Altitude-2730 M; Latitude- 34° 34' 0 N; Longitude-74° 45'0 E) Jammu & Kashmir, India was collected and brought to laboratory in cold-box in sterile condition. Yeasts were isolated from the soil by dilution-plate method. Initially, these samples were placed on potato dextrose agar (PDA) plates incubated at 6^{o}C for 30 days and purified cultures were maintained at 4^{o}C in the refrigerator on malt agar (MA) medium.

Growth characterization

The yeasts were characterized for their growth behavior in response to change in temperature and pH. The inoculated PDA plates were kept at 4^{o}C, 20^{o}C, 30^{o}C and 37^{o}C for ten days. Effect of pH on growth was tested on PDA medium with different pH 3, pH 4, pH 5, pH 7, pH 9 and pH 11. The selection was made on the basis of the ability (psychrotolerant) or inability (psychrophilic) of selected isolates to show normal growth above 20°C.

Screening of amylase producing yeast

The yeast isolates were inoculated on to screening medium 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. The plates were incubated at 4^{o}C for 15 days. Amylolytic activity were determined by the development of a colourless halo zone surrounding yeast colonies when flooded with Lugol solution (1% iodine in 2% potassium iodide, w/v) to the plates [27].

Taxonomic identification of yeast strains

The colony and cell structure of PT1 and SPY11 were analyzed according to the method given earlier [28]. Molecular identification of PT1 and SPY11 was performed on the basis of sequence characteristics of D1/D2 domain and ITS and 5.8s region of the large rDNA, respectively. DNA isolation from yeast was performed as per the protocol given earlier [29]. Sequences were compared with the non-redundant NCBI database using BLASTN, with the default settings used to find the most similar sequences, and were sorted by the E-score. A representative sequence of 10 most similar neighbours was aligned using CLUSTAL W2 for multiple alignments with the default settings. The multiple-alignment file was then used to create phylogenetic trees applying UPGMA method in MEGA5 software.

Enzyme production and extraction

For the production of enzyme, a 72hrs old culture (cell density of 10^6 ml^{-1}) of yeast isolate PT1 and SPY11 grown on YM medium were transferred to 250ml flask containing: (g l^{-1}) $(NH_4)_2SO_4$-

5g, KH_2PO_4-1g, $MgSO_4$-500mg, $CaCl_2$-100mg, $NaCl_2$-100mg, $FeSO_4.7H_2O$-0.01, soluble starch (Sigma)-10g, pH adjusted to 6.0.The inoculated medium were incubated at 12°C at 150 rpm,after every 24h over a period of 5 days, the culture was harvested to measure enzyme activity. The cells were spun down at 5000 rpm for 10 minsupernatant was saturated with $(NH_4)_2 SO_4$ to 50 % level and precipitated proteins were collected by centrifugation at 10000 rpm for 10 min. After dialysis against 100 mM phosphate buffer (pH 7.0) at 4°C, protein was re-suspended in the same buffer. The dialyzed solution was used as the source of α-amylase for the catalytic properties.

Protein determination

Total protein determination was performed according to [30]using bovine serum albumin as standard.

Determination of α-amylase activity

To 1 ml pre-cooled(1%) soluble starch solution, 1 ml of properly diluted enzyme (kept at 4°C) was added; the reaction-mix was incubated at 15°C for 30 min. A 0.3ml aliquot of this reaction-mix was transferred to new test tube, and 0.3ml of 3,5-dinitrosalicylic acid reagent was added to it. The solution was boiled for 5min, cooled down to room temperature and then 2.7ml of distilled water was added to it. 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 [30]. The experiments were performed in three sets of duplicate cultures, and the mean values of soluble glucose equivalent released by enzyme were determined.

Enzyme characterization

Temperature as well as pH optima of α-amylolytic enzymes produced by PT1 (psychrotolerant) and SPY11 (psychrophilic) yeast isolates were investigated. The temperature optimum of the enzymes was evaluated by measuring the α-amylolytic activity at different temperatures (4°C, 25°C and 40°C) in 100 mM sodium phosphate buffer (pH 7.0).The α-amylolytic activity were investigated by varying thepHin the range of (4.0 to 11.0)using following buffers (100mM): sodium acetate buffer (pH 3.0-5.5), sodium phosphate buffer (pH 6.0-7.0), Tris–HCl buffer (pH 7.5-9.0) and glycine-NaOH buffer (pH 10.0-11.0).

Statistical analysis

All measurements were performed three times (n=3) using the same sample, and statistical analysis was performed manually. Statistical significance of the means was evaluated using one way analysis of variance. Subsequent comparisons were performed using the LSD test. Differences were accepted as significant when $P < 0.05$.

RESULTS

Isolation and screening of yeast for amylase activity

Two yeast morphotypes were isolated from the soil of Baramullah (Altitude-2730 M; Latitude-34° 34' 0 N; Longitude-74° 45'0 E) Jammu & Kashmir, India. These yeast isolates (PT1 & SPY11) were found most potent to secrete extracellular amylase on the plates under near optimal temperature and pH condition. PT1 (psychrotolerant) and SPY11 (psychrophilic) were picked up as pure cultures showing normal and defective growth, respectively, above 20°C.The strain PT1 was psychrophilic yeast, because it can grow at 0-5°C on lactose as a sole carbon source, but can't grow at 20°C. Psychrophilic yeasts grow below 5°C and exhibit no growth above 20°C [31]. PT1 and SPY11 were selected for enzymatic study on the basis of their promising character with high potential of amylase enzyme production.A colourless halo zones around yeast colonies were developed when flooded with Lugol's solution on plates showing that both yeasts secrete starch-digesting enzymes[27]. Six psychrophilic fungal isolates has been reported earlier from the new geographical region of Jammu and Kashmir as a source of cold-active pectinolytic activities of oenological grade [32]. Earlier, [33]favored 20°C to be cardinal temperature to

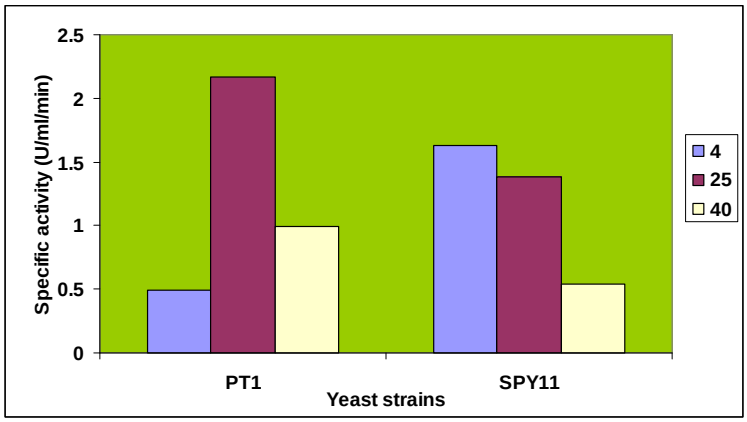
delimit psychrophiles. But [34] considered the organism showing residual growth at 20°C also to be psychrophile.

Morphological and molecular identification of yeast strains

The isolated yeast strains PT1 and SPY11 were morphologically as well as molecularly identified as reported earlier [35, 36].

Effect of temperature on enzyme activity:

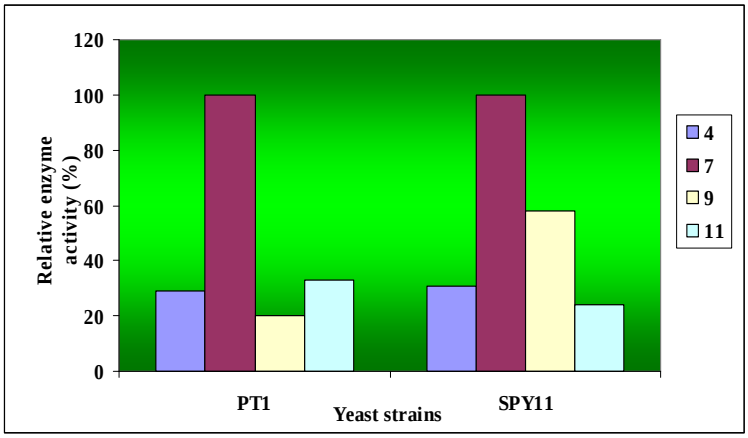
The *Rhodotorulamucilaginosa*PT1exhibited maximal specific α-amylase activity 2.17 U/ml/min at 25°C and there was dramatically decrease in α-amylolytic activity at 4°C and 40°C.which is consistent with the feature of the cold-adaptive enzymes [24].In case of psychrophilic yeast strain *Cystofilobasidiumcapitatum* SPY11 maximal specific amylase activity 1.63 U/ml/min was found at 4°C reflecting the nature of cold-active enzymes. However with increase in temperature above 25°C there was sharply decrease in enzyme activity (Graph 1).Cold-active yeast β-galactosidases that are able to degrade lactose at low temperature and having maximal enzyme activity at 4°C have been reported [37]. A cold active α-amylase from Antarctic psychrophile*Alteromonashaloplanktis*was reported to exhibit maximum α-amylase production at 4°C[38, 39].



Graph 1. Effect of different temperatures on amylase activity.

Effect of pH on enzyme activity:

Both *Cystofilobasidiumcapitatum* SPY11 and *Rhodotorulamucilaginosa*PT1showed maximal α-amylase activity (100%) at pH 7.0. The α-amylase from both strains was neutral in nature, although 58% of second peak activity of*Cystofilobasidiumcapitatum* SPY11α-amylase was found at pH 9 (Graph 2).As previously reported from Antarctic psychrotroph, *Alteromonashaloplanctis*A23, in which more than 60% of the optimalamylase activity was retained between pH 6.0 and 8.5 [21].Thus α-amylase secreted by *Cystofilobasidiumcapitatum* SPY11 and *Rhodotorulamucilaginosa*PT1 showed optimal activity at pH 7.0.It has been reported earlier that two yeast isolates with β-galactosidaseactivities ranging from pH 4.0-11.0, but maximal relative enzymatic activity was found at pH 4.0[37].



Graph 2. Effect of different pH values on amylase activity.

DISCUSSION

Amylases are one of the most important industrial enzymes finding application in a variety fields.As to the cold-active amylases, hardly any class of microbes other than bacteria of Antarctica origin has been explored [40].Therefore, cold-active amylases from these bacteria have become the model for biochemical studyand contemplating future applications[40]. Cold-active enzymes with unique molecular adaptabilitieshave opened up potential newer areas of applications [41, 32, 36]. The both yeast isolates possess potential to secrete amylases at optimal pH and temperature.

The*CystofilobasidiumcapitatumSPY11* were psychrophilic yeast whereas*Rhodotorulamucilaginosa* PT1 were psychrotolerant yeast. Earlier one being psychrophilic in nature secreted cold-active α-amylase but *Rhodotorulamucilaginosa* PT1 secreted cold-adaptive α-amylase under optimal conditions. The psychrotrophic yeasts with β-galactosidase activity have been isolated earlier [42]. The optimal pH of the α-amylases secreted by *Cystofilobasidiumcapitatum*SPY11 and *Rhodotorulamucilaginosa* PT1 is 7.0. It has been previously reported that in general acidic yeast-amylases possess optimum pH usually in the range of 4.0 and 6.0[8, 13, 43, 44]. This unusual pH improves robustness of an enzyme and makes its features more attractive in form of various industrial applications.

The cold-active pectinolytic enzymes (Pectin methylesterasePME, endo-PG and exo-PG) from the newly isolated and identified psychrophilic yeast *Cystofilobasidiumcapitatum*SPY11 and psychrotolerant yeast *Rhodotorulamucilaginosa* PT1 that exhibited 50–80% of their optimum activity under some major oenological conditions pH (3-5) and temperatures (6 and 12°C) could be applied to wine production and juice clarification at low temperature [36]. The amylases secreted by psychrophilic *Cystofilobasidiumcapitatum*SPY11 and psychrotolerant*Rhodotorulamucilaginosa* PT1 yeast isolates can be used as a potential biocatalyst in different industries where processes are being carried at low and moderate temperature.

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