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Research Article

Thermo-tolerant cold-active amylolytic activity from a psychrotolerant yeast isolate, *Rhodotorula mucilaginosa* BPT1

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Abstract: Amylolytic activity from a psychrotolerant yeast *Rhodotorula mucilaginosa* BPT1 was assayed under various pHs and temperature conditions and in presence of various reagents and metal ions. The enzymes showed 32 % of residual activity at 20^{o}C, 28% at 4^{o}C and almost 90% stability at 4-20^{o}C in 1h. Most characteristically, the amylolytic activity was slightly enhanced during incubation at 50^{o}C boiling with two pick-activities each. The optimum pH for the enzymes was 7.0, but they yielded about 80% activity at acidic (5-6) and alkaline (9-11) range of pHs. The enzymes showed retention of about 80% of the activity after 1h of incubation at various pHs (3-11). While the amylolytic activity was enhanced by Ca^{2+} (125%), Mg^{2+} (200%), $Mn2^{+}$ (175%), and Cl^{-1} (120%) and was inhibited by Fe^{2+} (20%) and Cu^{2+} (80%). The enzymes retained 85% of activity after 1h of incubation with EDTA, while SDS was found to enhance (125%) their activity during the same period. Our result showed that the yeast secreted an ideal cold-active, metals, chelator and detergent resistant, and pH and heat stable amylolytic activity which may be suitable in detergent, textile and other industrial applications.
Keywords: Rhodotorula mucilaginosa BPT1, Amylolytic activity, Thermo-amplification, Enzyme characterization.

INTRODUCTION

Amylases (EC 3.2.1.11) are an important group of enzymes, enabling an organism to degrade polyglucosides with α-1,4 glucosidic bonds such as starch and glycogen. Industrially, amylases find applications in a variety of fields including those for starch-saccharification, foods, pharmaceutics and detergents [1], each application requires unique set of properties with respect to specificity, stability and tolerance to various physic-chemical conditions such as temperature, pH, metal, detergent, chelator etc. Screening of microbes with amylolytic activities, therefore, could facilitate the discovery of novel amylases for different purposes.

Cold-active enzymes have inbuilt potentiality to save energy and protect the processes from contamination and thus are attractive for the industries [2]. With the exception of best characterized cold-adaptive α-amylase from Antarctica bacterium *Pseudoalteromonas planktis* [3], there are only few reports on cold-active amylases from other bacteria [4], Actinomycetes [5] and earthworm [6]. The cold-active enzymes so far reported have been found to lack stability at moderate to high temperature [2] and such other features as required for their industrial applications.

Yeasts are unicellular fungi with ubiquitous distribution in many ecosystems. Recently, there have been great deals of interests in starch degrading yeasts due to their potentiality in biotechnological applications, such as a source of hydrolytic enzymes or the production of single-cell protein or ethanol from starchy biomass. Although yeasts are not among the industrial producers of amylases, the enzyme is widely distributed in many yeast species including *Lipomyces kononenkoae*, *Schwanniomyces alluvius*, *Trichosporon pullulans*, *Candida antarctica* and *Cryptococcus flavus* [7]. Despite these, only limited research has been directed towards the amylases produced by the *Rhodotorula* genus [7]. In the present communication, I report novel thermo-amplifiable amylolytic activity from a natural psychrotolerant yeast isolate, *R. muclaginosa* BPT1 and describe some of their biochemical properties of immense biotechnological importance.

MATERIALS AND METHODS

Isolation and maintenance of yeast

R. mucilaginosa BPT1 was isolated originally from the soil collected from Baramullah (located in 34.2^{o} N and 74.34^{o}E) India as described earlier [2, 8]. The yeast was maintained on ME slant at 4^{o}C, sub-culturing at regular intervals.

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°C, 20°C, 30°C and 37°C for ten days. Effect of pH on growth was tested on PDA medium with different pHs (pH 3, pH4, pH5, pH7, pH9 and pH11), since agar could not solidify at pH 3 and 4 it was replaced with psillium as gelling agent [9]. The plates were incubated at 20°C for five days.

Test for amylase production

The yeast isolate was 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°C for 15 d. Amylase production was detected after flooding the plates with lugol solution.

Enzyme production and extraction

The enzyme (amylases) was produced by transferring 1 ml culture (density 10^6 l^{-1}) from YM medium to 500-ml conical flask containing 100 ml of the yeast production medium (YPM: 0.67% YNB and 2% starch). The growth was carried out at 15°C on a rotatory shaker at 150 rpm, and was monitored by measuring OD_{600} of growth medium. At different time intervals, 5 ml of the samples were withdrawn and cells were spun down at 5000 rpm for 10 min. The supernatant 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 resuspended in the same buffer.

Protein content

Protein concentration was measured according to Lowry *et. al.* [10] using bovine serum albumin as standard and was stored at -20°C.

Determination of amylolytic activity

To 1 ml pre-cooled at 15°C of 1% 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 [11]. The experiments were performed in three sets of duplicate cultures, and the mean value of soluble glucose equivalent released by enzyme was determined.

Enzyme characterization

The pH optimum of the enzyme was determined by varying the pH of the reaction mixtures using the following buffers (100 mM): sodium acetate (pH 3.0-5.5), sodium
phosphate (pH 6.0-7.0) and Tris-HCl (pH 7.5-8.0). To determine the pH stability, the enzymes were pre-incubated in different buffers for 1h. The residual activity was assayed in 100 mM sodium phosphate buffer (pH7.0).

The temperature optimum of the enzyme was evaluated by measuring the amylolytic activity at different temperatures (4°C, 20°C, 40°C, 50°C) in 100 mM sodium phosphate buffer (pH 7). The effect of temperature on stability of amylolytic activity was determined by measuring their residual activity after pre-incubation in 100 mM sodium phosphate (pH 7.0) at 50°C for 1h, 2h, 3h, 4h and 24h. The protein was also tested for their stability at boiling temperature for 1min, 5 min, 10 min, 15 min, 20 min and 30 min

For determining the effect of metal ions on amylolytic activity, assay was performed after pre-incubation of the enzyme with various metal ions at a final concentration of 5 mM, at 15°C for 60 min. Likewise, enzyme was pre-incubated with EDTA or SDS to the final concentration of 5 mM and 1% (w/v) respectively at 15°C for 60 min, to see their effect on amylolytic activity. The activity of the enzyme alone in 100 mM sodium phosphate buffer (pH 7) was taken to be 100%.

Substrate specificity

Salted out amylolytic enzyme (0.5 ml) was incubated with 0.5 ml of Starch, Maltose, Glycogen, Amylopectin or Amylose (1% w/v) in 100 mM sodium phosphate buffer (pH7) at15°C for 30 min. The product was assayed by DNS method.

Taxonomic characterization of BPT1

Taxonomic characterization of BPT1 was carried out as described earlier [2, 8].

Reproducibility of result

All the experiments were carried out in triplicates and three times. Statistical significance of the means was evaluated using one-way ANOVA manually. Differences were accepted as significant when $P < 0.05$ for all experiments except for the effect of high temperature and boiling. Seeing the unprecedented results of the effect of these factors (high temperature and boiling) statistical significance was ignored.

RESULTS AND DISCUSSION

The psychrotolerant yeast BPT1 was found to grow optimally in the temperature range of 20°C-30°C and pH range of 3-7(Table 1).

Table-1: **Colony growth of BPT1 under selected growth conditions**

	Conditions	Growth of colony-diameter (mm)
pH	3	2
	7	2
	9	0.2
Temperature (^oC)	4	1
	20	2
	30	2
	37	-

During growth on agar plates containing starch as substrate, the yeast BPT1 formed a large digesting halo around its colonies after staining with lugol solution showing that this yeast secreted starch-digesting enzymes. The time course of amylase secretion and starch degradation by BPT1 during batch culture in a medium containing soluble starch (YPM) is shown in Figure 1. The degradation of starch was accompanied by cell growth and amylase secretion in the medium. The extracellular amylolytic activity increased during cell growth and reached a maximum value (2.16U ml^{-1}) at 96 h of incubation, which continued till 120 h (Figure1).

The amylolytic activity was maximum (100%) at 40^{o}C, went down to 32 % at 20^{o}C and 28 % at 4^{o}C. The enzymes lost ≈65% activity at 40^{o}C, though it showed not more than 10 % of erosion in its activity at other temperatures in 1 h incubation (Figure 2).

A much unexpected result was obtained with respect to their stability at 50^{o}C (Figure 3). The enzymes showed a unique phenomenon of thermo-amplification of activity during incubation for various periods at 50^{o}C and during boiling. There were two peak-activities, one moderate (~ 130 %) at 1h and another high peak (~ 250%) at 2h when incubated at 50^{o}C (Figure 3). The enzyme did show two pick activities of 250% and 300 % at 15 min and 30 min of incubations respectively (Figure 4) during boiling. The experiment was repeated ten times but almost similar trend was obtained, some variation was found with respect to second peak activity which was obtained 6 out of 10 times.

The optimal pH for the enzymes activity was found to be 7.0, 80% of the activity was retained at alkaline (9 and 11) and about 60% at acidic (5 and 6) pHs (Figure 5). The enzymes showed nearly 80% activities after 1h of incubation at various pHs.

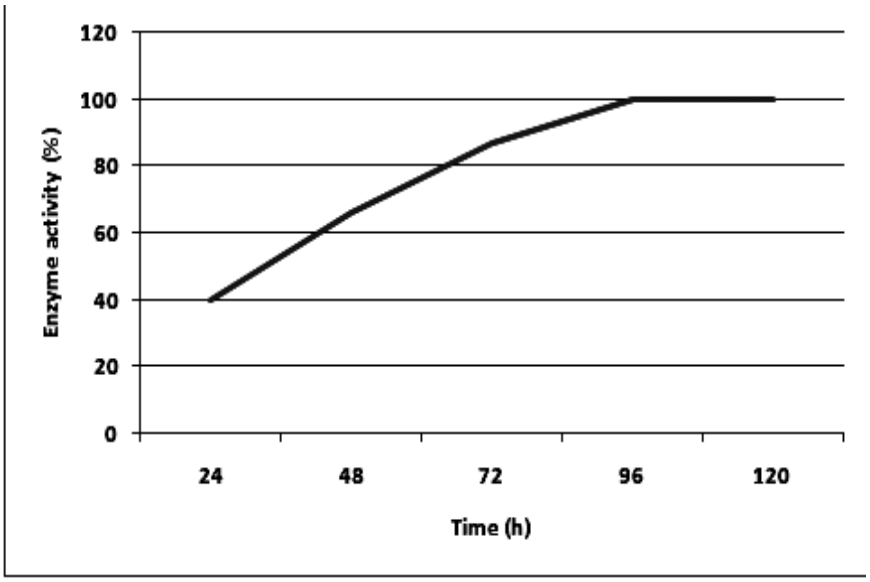

Fig-1: Amylolytic activity in BPT1 amylase production broth as function of time

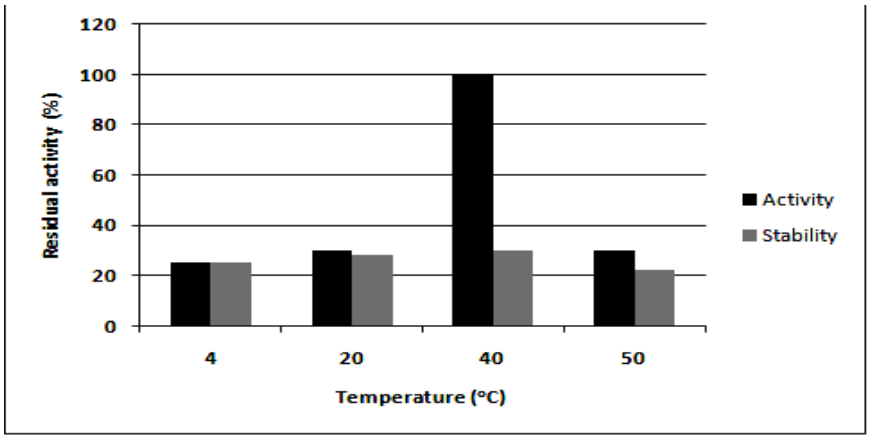

Fig-2: Effect of temperature on the amylolytic activity from BPT1

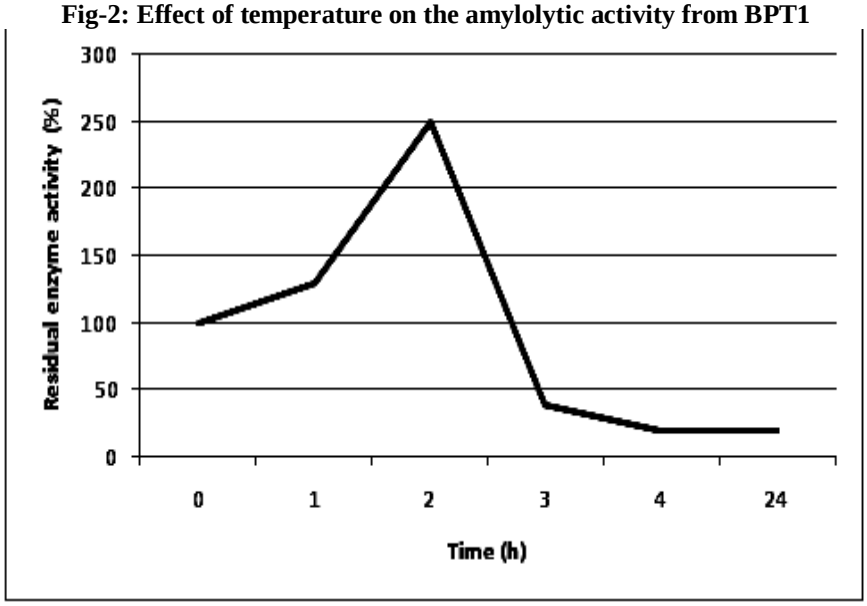

Fig-3: Enzyme activity at 50°C as a function of time

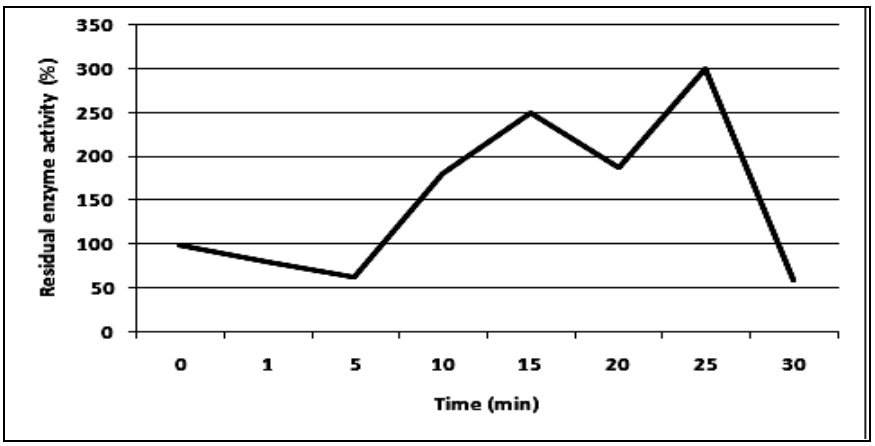

Fig-4: Effect of boiling temperature on the amylolytic activity from BPT1

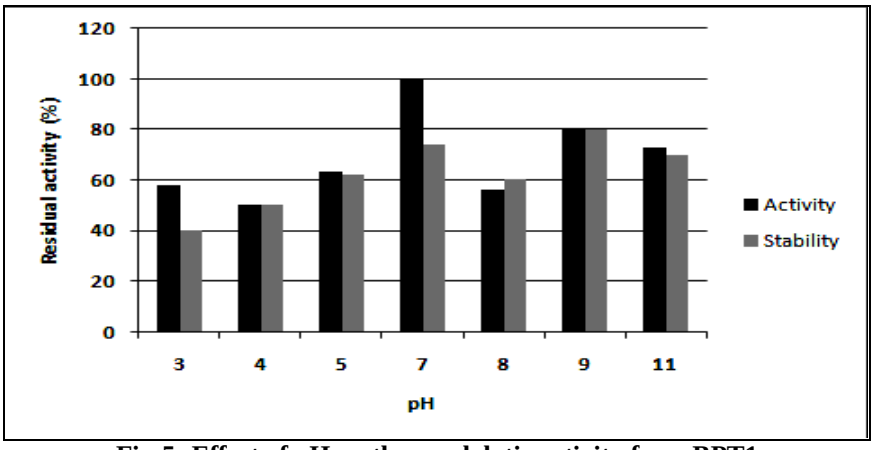

Fig-5: Effect of pH on the amylolytic activity from BPT1

Amylases have been reported from a variety of yeast strains [7], there has hardly been any report of their isolation from *R. mucilaginosa*. Amylases are one of the most important industrial enzymes finding application in a variety fields. Bacteria are the most important organisms serving the source of industrial grade amylases so far, though enzymes with unique set of biochemical characteristics fit for specific applications have been reported from other classes of microbes. As to the cold-active amylases, hardly any class of microbes other than bacteria of Antarctica origin has been explored [3, 4]. Therefore, cold-active amylases from these bacteria have become the model for biochemical study [3] and contemplating future applications. The amylolytic activity with utterly novel features from a yeast-isolate, BPT1 isolated from new geographical region is reported.

Physiologically, BPT1 showed some variance in carbon and nitrogen utilization profile [8] as compared to that of the CBS strains of *R. mucilaginosa* though it shared hundred percent similarity in the D1/D2 sequence of rDNA with many strains of this yeast available in GeneBank database.

The BPT1 amylolytic activity showed optimum activity at 40^{o}C at pH 7.0 which is not in agreement with the data for the α-Amylases from for *S. alluvius* ATCC 26074, *S. alluvius* UCD 54-83, *L. kononenkoae*, *C. antarctica* CBS 6678 and *C. flavus* [7]. The enzymes retained nearly 80% of their activity at 4^{o}C and 20^{o}C. They are comparatively thermostable at lower temperatures. At higher temperature, they behaved differently from all the amylases reported so far. Initially, they showed moderate enhancement (130%) in activity at 50^{o}C, but then after a sudden spurt (250%) in enzyme activity was found. The thermo-amplification was more evident during boiling of the enzyme yielding the pick activities of 250 % (15 min) and 300 % (25 min). This attribute is very difficult to be explained in light of general concept of very unstable cold-active enzyme. It needs more data to conclude whether it is an adaptive feature of cold-active enzyme enabling a psychrotolerant microbe to modulate the rate of metabolism under two different growth conditions.

The optimal pH of the enzymes is 7.0 which is again a deviation from general acidic yeasts-amylases with optimum pH usually in the range of 4.0 and 6.0 [7]. Moreover, more than 80% 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.

The amylolytic activity from BPT1showed only a moderate stimulation (125%) by Ca^{2+} but the latter could not sustain the activity for next 1 h (Table 2). Unlike this, the activity was both induced (120%) and sustained by Cl^{-1} during next 1 h of incubation. The activity was most significantly stimulated by Co^{2+} and Mg^{2+} (upto 200 %), but the activation could not be maintained to this level during next 1 h of incubation with these metal ions. Whereas the activity stimulated by Mn^{2+} (175%) was not only sustained but further enhanced (by 80 %) during next 1h- incubation. On the other hand, the activity was inhibited moderately (20%) by Fe^{2+} to drastically (80%) by Cu^{2+}. The amylolytic activity was not affected by EDTA but was stimulated (120%) by SDS during 1h of incubation.

Substrate specificity

The ability of amylolytic activity on selected substrates is given in Figure 6. The enzyme could degrade starch and to a very lesser extent glycogen (about 20%) and amylopectin (about 10%).

Table-2: Effect of various compounds on the amylolytic activities from *R. mucilaginosa* PT1.

Compounds (4 mM)	Activity Relative activity (%)	Stability Relative activity (%)
Control	100	75
$CaCl_2$	125	75
$MnSO_4$	170	250
$MgSO_4$	175	100
$ZnSO_4$	100	70
$CoCl_2$	200	130
$FeCl_2$	80	80
$CuSO_4$	20	10
NaCl	120	120
EDTA	100	85
SDS	100	120

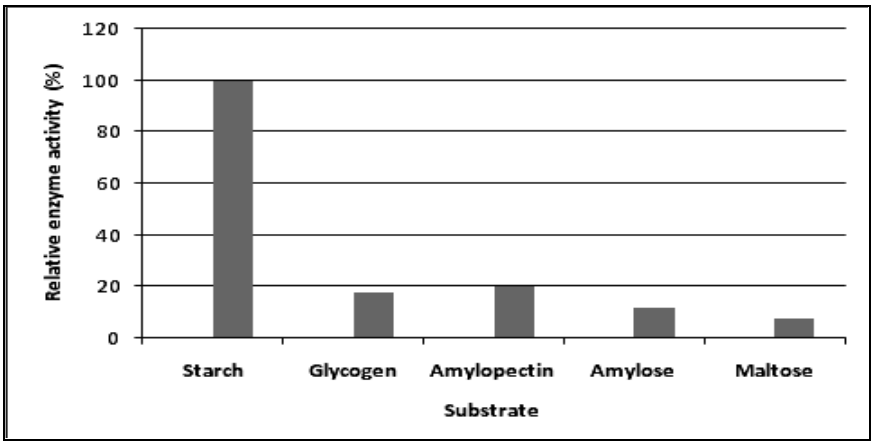

Fig-6: BPT1 amylolytic activity on different substrate

The yeast was characterized morphologically, physiologically and molecularly and assigned to the genus *Rhodotorula* spp. The D1/D2 sequence of BPT1 showed 100 % similarity to type strain and many of other strains of *Rhodotorula mucilaginosa* in the GenBank database. Therefore, the isolate was named *R. mucilaginosa* BPT1 [2,8]. The nucleotide sequence has been deposited in the GenBank database under accession number JNO91167

As reported from studies on other yeast amylases, the enzyme was moderately to highly inhibited by metal ions. The results indicated that the amylolytic activity from *R. mucilaginosa* PT1 was not affected by most of the metal ions tested (Co^{+2}, Mn^{+2}, Mg^{+2}, Zn^{+2} and Ca^{+2}) except Cu^{+2} (80%) and Fe^{2+} (20%). The amylolytic activity was rather stimulated by many of these ions (Co^{+2}, Mn^{+2} and Mg^{+2}) or even stabilized by one of them (Mn^{+2}) since the activity was enhanced during incubation with it. Among these metals, Co^{+} and Mg^{+} have earlier been reported to enhance the activity in bacteria while Cu^{+} and Fe^{+} are general inhibitors of amylases [12]. 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 enzyme is not a metalloenzyme. In most of the cases, amylases have been found to be Ca^{+2} dependent metalloenzyme [13], though Ca^{2+} independent amylases have also been reported [4]. The enzyme activity was inhibited in the presence of Cu^{+2} and Fe^{+2}, these ions have also been reported to inhibit other amylases produced by yeasts [4]. The amylolytic activity was most surprisingly enhanced by SDS indicating that hydrogen bonds may not play a key role in maintaining enzyme activity [4]. 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 [4].

The biochemical properties of BPT1 amylolytic activity, therefore, differ in a great deal from all the previously reported amylases. Preliminary biochemical characteristics suggest that these activities may find application in laundary detergent and textile industry. Earlier, *R. mucilaginosa* has been reported to produce arthrospore [8] rare cold-active enzyme [14], β-carotene [15], xylitol and ethanol [16] and have potential to be applied to bioremediation of oil [17]. Association of this yeast with diseased lesions [8], however, may be a hindrance in the way of its industrial application. 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. The work also highlights exploration of microbes in the cold-regions [18] apart from Antarctica [3, 4] and deep ocean [19] to find important source of cold-active industrial enzymes. Moreover, this is the first report of a stable cold-active amylolytic activity from yeast with potential biotechnological applications.

CONCLUSION

R. mucilaginosa amylases possess desired level of tolerance towards temperature, pH and metals and seem to be highly potent for various industrial applications. The strain *R. mucilaginosa* BPT1 has some variation at physiological level as compared to reported strains thus indicating a higher level of adaptability that has enabled this yeast to occupy wider variety of niches. The present findings call for further work on this yeast and its amylases to exploit it industrially.

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