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RESEARCH ARTICLE

COLD ADAPTED AMYLASE AND PROTEASE FROM NEW *STREPTOMYCES* 4ALGA ANTARCTIC STRAIN

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Abstract
The ability of a new polar strain coded *Streptomyces* 4Alga, isolated from vegetation samples from East Antarctica, to biosynthesis cold adapted amylases and proteases was investigated. Thermal inactivation studies shown that alpha-amylase enzyme retained almost 90% and 80% of its activity at optimum temperature (30°C) during the interval of 20-60 minutes of incubation. Instead, after 20 minutes of incubation at low temperature (20°C) alpha-amylase activity decreased. At optimum temperature beta-amylase retained almost 80% of its activity after 50 min of incubation. At 20°C beta-amylase showed 60% relative activity after 60 min of incubation. While, protease retained approximately 75% of its activity at optimum temperature, at lower temperature seamed to be less stable after one hour of incubation. The reported enzymes may have wide spread application for detergent and pharmaceutical and food industry.

Keywords: *Streptomyces sp*., cold-adapted enzymes, amylase, protease

Introduction

Psychrophilic and psychrotrophic microorganisms have been defined as organisms able to grow at temperatures close to 0°C, but displaying different upper growth limits around 20°C for the former and around 40°C for the latter. The distinction, not really scientifically justified, is essentially useful to select the more appropriate organisms for specific studies. It is clear that as far as molecular adaptation to cold is concerned, the interest is to select an organism displaying extreme properties, meaning an upper growth limit as low as possible (Gerday et al. 1997).

Temperature is one of the most important environmental factors for life. Cold-adapted or /or eukaryotic and represent a significant portion of the living world because temperatures over a considerable portion of our planet (e.g. polar and alpine regions, deep-sea waters) are below 5°C. Evolution has allowed these adapted organisms, named psychrophiles, to survive and grow in the restrictive conditions of these cold habitats. In these environments psychrophiles display metabolic fluxes more or less comparable with those exhibited by mesophiles at moderate temperatures (D'Amico et al., 2001).

The specific application of amylase and protease that have optimal activity at extreme temperatures are widely used in household detergents and in the food processing, animal nutrition, textile, pulp and paper, leather processing, and chemical industries (Syed et al., 2009; Lazim et al., 2009; Ahmed et al., 2008). For each application, the enzymes have to fulfil numerous requirements related to features such as activity and stability,

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substrate specificity and enantioselectivity (Podar and Reysenbach, 2006).
The possibility of using actinomycetes especially, *Streptomyces* for enzyme production has recently been investigated (Syed et al., 2009). The streptomycetes, abundantly found in soil and vegetation, are a well-known bacterial group that are especially important because of their capacity to produce antibiotics and several classes of enzymes and enzyme inhibitor (Azeredo et al. 2003; Ghadin et al., 2008; Chakraborty et al., 2008).
The present study was carried out to study the ability of a new polar strain, coded *Streptomyces* 4Alga, to grow in cold conditions and to produce amylase and protease cold active and to investigate enzymes activity and thermal stability inactivation at optimum temperature and at low temperatures.

Materials and methods

Chemicals: AZCL-Amylose and AZCL-Casein were purchased from Megazyme International Ireland Ltd., Ireland.

Microorganism: The new polar streptomycete strain coded *Streptomyces 4Alga* was isolated from Antarctic vegetation sampled from East Antarctica (Progress Lake 2). This strain was included in Collection of BioFood Platform, acronym MIUG (Industrial Microbiology Laboratory University of Galati, Romania) (Cotarlet et al. 2008).

Conditions of cultivation and crude extracts acquiring
The stock cultures were maintained at 20°C on Gause-agar medium containing (%): 2.0 potato starch, 0.05 K_2HPO_4, 0.05 $MgSO_4 \cdot 7H_2O$, 0.1 KNO_3, 0.05 NaCl, 0.001 $FeSO_4 \cdot 7H_2O$, 2.5 agar, pH 7.2–7.4. For enzymes production the *Streptomyces* 4Alga strain was cultivated in nutrient liquid medium containing (g/L): soluble starch 20.0, corn steep liquor 10.0, $(NH_4)_2SO_4$ 6.0, $CaCO_3$ 8.0, NaCl 5.0 and soybean oil 0.2 mL, pH 7.0 on a rotary shaker (230 rpm) at 20°C, for 10 days of cultivation (Cotarlet et al. 2008). The crude extracts were obtained after cultivation in submerged conditions and biomass separation at 6000 rpm, for 15 minutes.

Amylase and protease assays
The amylolitic and proteolytic activities in crude extract were monitored at low temperatures (10, 20°C) and optimum temperature for each enzyme, by using a adapted method for alpha-amylase, Merck method for beta-amylase assay, and for protease a modified Anson method was achieved.
Alpha-amylase assay based on a selective distinction of the hydrolysis products in 0.1 N Lugol solution was determined. One α-amylase unit according to this method is defined as the amount of enzyme which generates a 0.05 decrease in the optical density, for 1 min, measured OD_{610} nm, of the colour iodine-starch complex, into a 1% starch solution, at pH 7.0, and different temperatures analysed (Bahrim et al., 2007).
Beta-amylase assay using Merck method was achieved. One β-amylase unit represents the amount of maltose (in mg) produced by 1 ml crud extract by using 1% starch as substrate, at pH 7.0, for 1 min, at different temperatures analysed. In the direction of quantify the maltose the Shaffer-Somogyi method was used with few modifications (Ranganna, 1977).
Proteolytic assay was determined via modified Anson method using 2% casein as substrate (Anson, 1938; Cupp-Enyard, 2008) and protease activity was expressed as Anson units. One Anson unit is the amount of enzyme which, under the analytical specified conditions (2 % casein as substrate, pH 7.0; for 15 min, at different temperatures analysed) hydrolyzed the casein at a speed that facilitates release, in one minute, the hydrolysis products soluble in the trichloroacetic acid; this provides coloration equivalent, measured at OD_{670} nm, to 1 µmol of tyrosine, in the presence of the Folin-Ciocalteau reagent by using a tyrosine standard curve over the range 0.02-0.24 µmol/mL (Folin and Ciocalteau, 1929).

Streptomyces 4 Alga characterization relating to the ability to produce hydrolase's cold-adapted
The tests were conducted on solid media supplemented with insoluble chromogenic substrates. Therefore, basal media, containing 2% agar at pH 7.0, was supplemented by adding 0.05% AZCL-Amylose or AZCL-Casein. Subsequently, wells with 0.5 cm diameter size were prepared into the mass of agar medium, and

after that were added 30–35 µL crude extract. The Petri plates were incubated at different temperatures 5 ºC, 15 ºC, 20 ºC, 28 ºC, 37 ºC and 50ºC. By substrate hydrolysis a blue circle zone was development around the wells. Substrate hydrolysis zone was measured and express in centimetres.

Thermal stability of amylase and protease from polar strain

To determine the effect of low temperature on amylase and protease activities, the reactions were carried out at 10ºC and 20°C and also at optimum temperature corresponding to each enzyme.

For establishing the thermal stability at optimum temperature and at low temperature, first the crude extracts were incubate in phosphate buffer pH 7.0 (1:1) at 20°C, for 15 min, and then the residual activity was determinate at low temperature and also at optimum temperature for each enzyme. Residual activities were expressed as % relative activity as compared to control (100% relative activity).

Results and discussion

There are many reports on isolation of amylase and protease enzymes from genus *Bacillus*, but very few reports on isolation of amylase enzyme from cold-adapted Antarctic streptomycetes (Chakraborty et al. 2008).

Novel filamentous bacteria coded 4 Alga isolated from vegetation samples from East Antarctica were genetically and biochemically characterized in order to establish the phylogeny and their ability to grow at lower temperature and to generate amylase and protease cold-actives. The results of the 16S rRNA gene sequence shown that this strain is 100% identical to sequences of *Streptomyces* sp. isolates from Norway and from Solomon Islands (in press).

Streptomyces 4 Alga characterization relating to the ability to produce hydrolase's cold-adapted

The hydrolytic potential of new polar strain *Streptomyces* 4Alga to produce different hydrolase's was evaluated based on insoluble chromogenic substrates (AZCL-Amylose, AZCL-Casein) hydrolysis at different temperatures (Fig. 1).

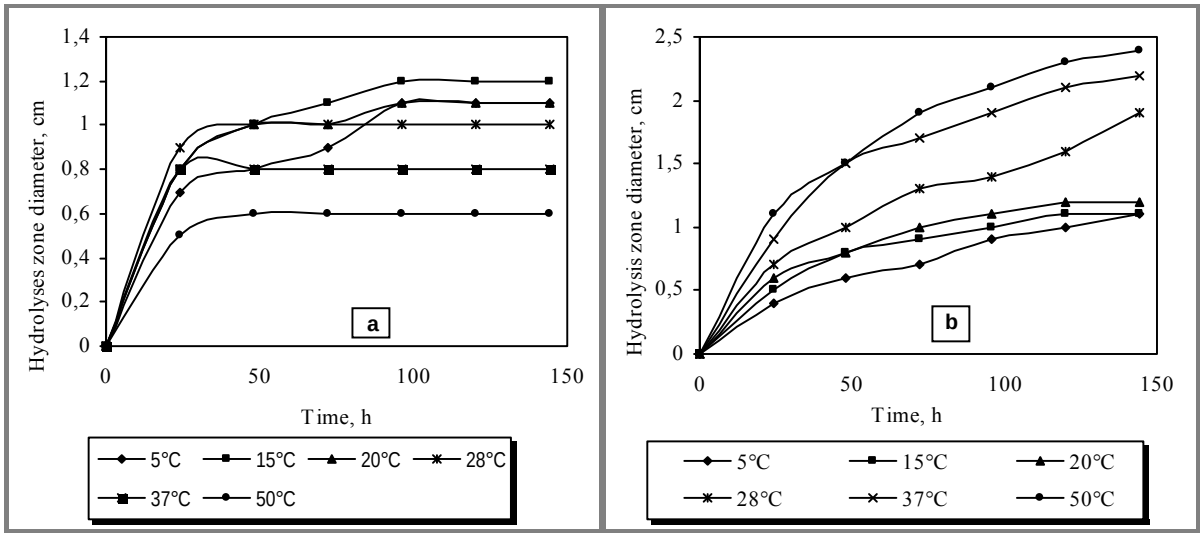

Fig. 1. *Hydrolyses potential at different temperatures on specific media supplemented with AZCL-Amylose (left), AZCL-Casein (right).*

The results certify that *Streptomyces* 4 Alga strain is able to produce amylase active at low temperature (5°C, 15°C and 20°C), the maximum hydrolytic yield being detected after 96 h of enzyme action. In protease case the maximum enzymes releases had been registered at 50°C, the biggest hydrolyze rate being developed after 144 h of incubation.

Effect of low temperatures on amylase and protease activities

Temperature had a significant effect on amylase and protease activity (Fig. 2). The activities of amylase and protease generated by the crude extract in order to establish the influence of lower temperatures (10°C and 20°C) comparing with optimum temperature of enzyme activity were studied.

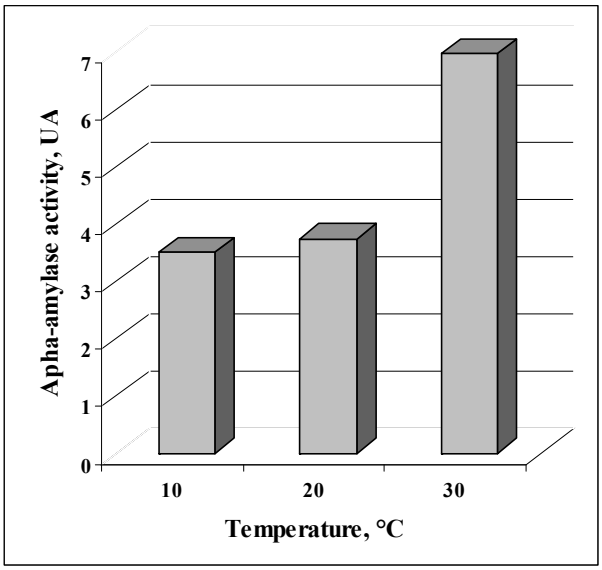

Fig. 2. *Alpha-amylase activity of crude extracts variation at low temperatures. The reaction was performed with 1% starch, at pH 7.0 for 10 min. The data are the average of three independent assays.*

Streptomyces 4 Alga exhibit relative high alpha-amylase activity at 10°C and 20°C respectively 3.51 and 3.75 UA, (50.21 % and 53.64 % respectively), comparatively with 6.99 UA, activity at optimum temperature at 30 °C (Fig. 2).

Extracelullar beta-amylase generated by *Streptomyces* 4Alga strain showed the highest activity at 30°C. A ratio of 85% and 60% respectively of the beta-amylase activities could still be detected at 20°C and 10°C (Fig. 3).

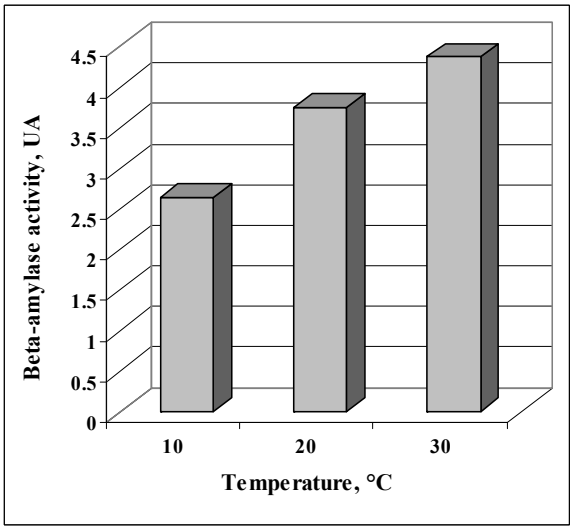

Fig. 3*. Beta-amylase activity of crude extracts variation at low temperature. The reaction was performed with 1% starch at pH 7.0 for 15 min. The data are the average of three independent assays.*

Most cold-active proteases display optimum activity at 30...45°C. Other authors reported temperature optima of 25°C and 20°C, respectively, for proteolytic activity. However, some psychrophilic bacteria produce proteases with optimum activity at 50°C or 60°C (Margesin et al. 2005). The optimal temperature activity for of protease from *Streptomyces* 4 Alga was established at 70°C (Cotarlet et al. 2009).

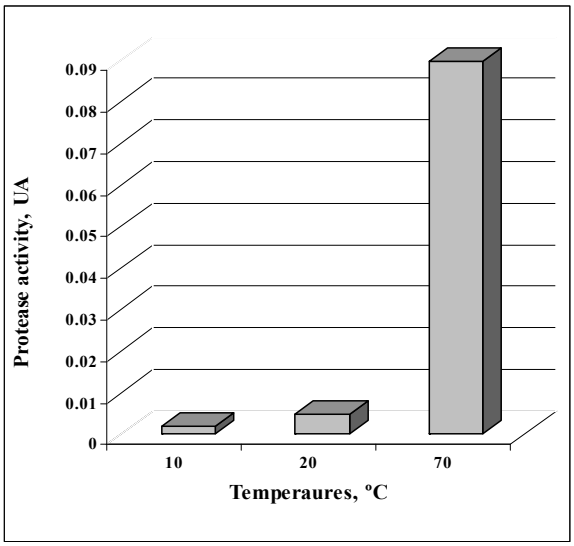

***Fig. 4**. Protease activity of crude extracts variation at low and optimum temperature. The reaction was performed with 1% starch at pH 7.0 for 15 min. The data are the average of three independent assays.*

Therefore, the activity of protease at lower temperatures (10°C and 20°C) was inferior comparatively with the proteoltytic activity registered at optimum temperature. Approximately 97% and 94% of protease activities were lost at low temperatures compared with the proteolytic activity at optimal temperature (Fig. 4).

Thermal stability of amylase and protease from polar strain

The amylase enzyme was found to be stable at pH 7.0 (phosphate buffer) when incubated for 1 h. There are various papers dealing with amylase having stability at pH 7.0. Chakraborty et al. 2008, mentioned novel α-amylase enzyme from marine *Streptomyces* sp. D1. stable in phosphate buffers.

Alpha-amylase retained almost 90% and 80% of its activity at optima temperature after 20-60 min of incubation at 20°C. The alpha-amylase from *Streptomyces* 4Alga isolate was able to retain almost 50% of its activity after 20-60 min of incubation at 20°C (Fig.5). Instead, after 20 minutes of incubation at low temperature alpha-amylase activity decreased.

At optimum temperature beta-amylase retained almost 80% of its activity after 50 min of incubation. Extracelullar beta-amylase showed 60% relative activity at 20°C after 50-60 min of incubation (Fig.6).

Whereas, protease retained approximately 45% and 75% respectively of its activity at optimum temperature (70°C) after 20-60 min of incubation and seamed to be less stable at lower temperature, 30% of initial activity after one hour of incubation (Fig. 7).

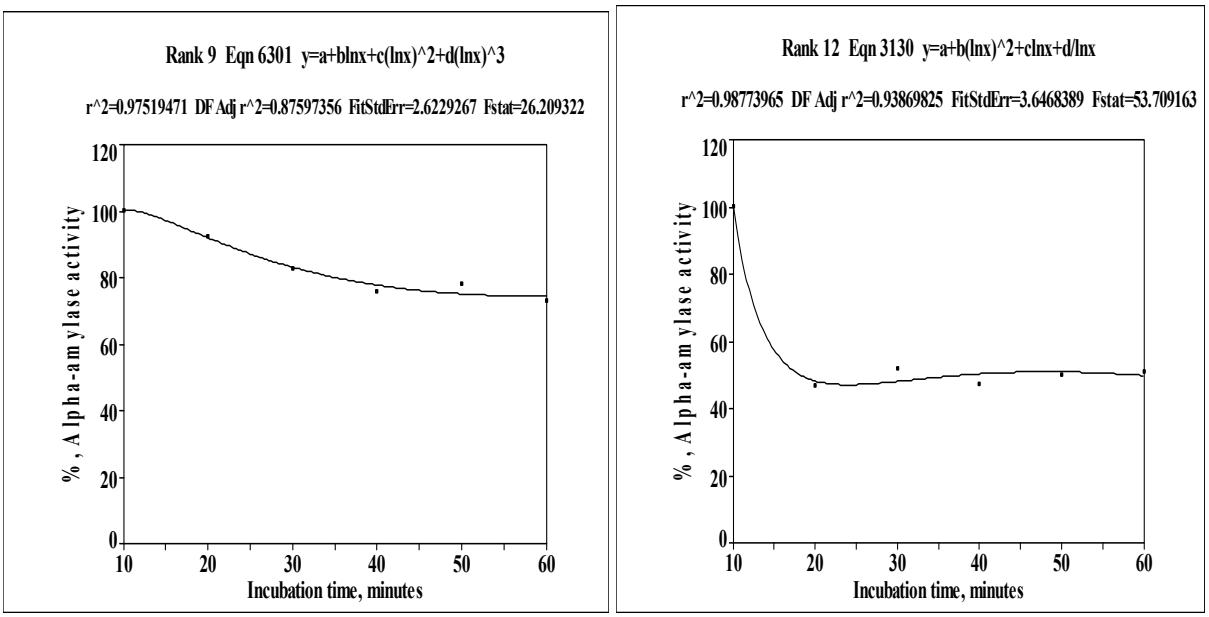

Fig. 5. *Thermal stability of crude extract alpha-amylase of Streptomyces 4 Alga at optimum temperature activity (30°C) (left) and at 20°C (right).*

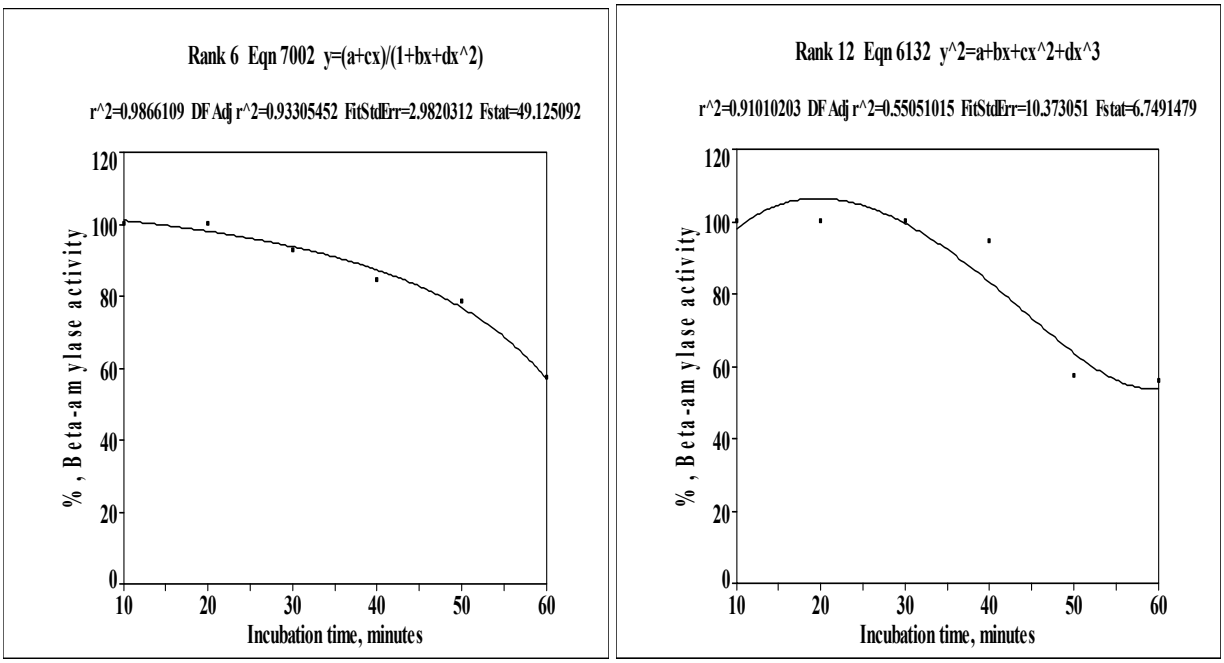

Fig. 6. *Thermal stability of crude extract beta-amylase of Streptomyces 4 Alga at optimum temperature (30°C) (left) and at 20°C (right).*

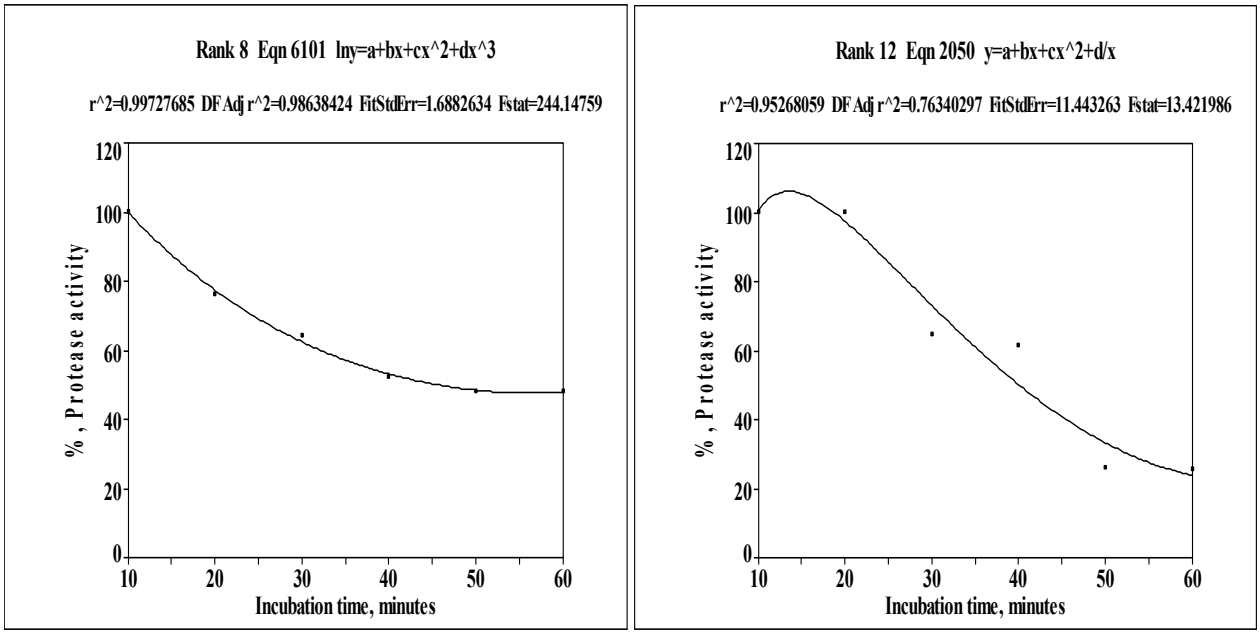

Fig. 7. *Thermal stability of crude extract protease of Streptomyces 4 Alga at optima temperature (70°C) (left) and at 20°C (right).*

This data attest the ability of the *Streptomyces* 4 Alga isolates to produce cold-adapted amylase and protease. The reported enzymes with the desired properties being active and stable at low temperature have potential relevance in molecular biology, food processing and bioremediation in cold climates (Margesin et al. 2005; Chakraborty et al. 2008).

Conclusions

The Streptomyces 4 Alga is an excellent filamentous bacterial strain able to produce amylase and protease adapted at low temperature (10°C and 20°C). The amylase displayed optimum activity at 30°C and good activity at 20°C and lost 40% of activity after 30 min of incubation at 20°C. Instead, the protease is active at higher temperature (optimum temperature at 70°C) and showed thermolability by incubating at 20°C, pH 7.0, during 15 minutes. There are various application fields of cold-active, thermolabile proteases. For example, such enzymes could be useful new tools in molecular biology, food processing and bioremediation. Amylase adapted at low temperature can be valuable sources in detergents, food industry and in bioremediation process.

Acknowledgements

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