Biotechnological conditions of amylase and protease complex production and utilization involving filamentous bacteria

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Abstract
Streptomyces, bacterial filamentous, largely spread into soil micro biota, have a high enzymatic potential, and are able to produce hydrolytic enzymes with natural implications in organic compounds biodegradation and bioremediation. Studies proved the variability of the biosynthesis potential and of the catalytic behaviour of the enzymes from the category of amylase and protease, synthesized by two selected strains of *Streptomyces* species, taken from local and East Antarctic coast soils. The preliminary researches show that the polar strain *Streptomyces* 11P features biosynthesis properties and the characteristics of the amylases and proteases synthesized, slightly modified comparing to strain *Streptomyces* MIUG 4.116, isolated from Romanian soil.

Key words: cold activity of enzymes, Antarctic soil micro biota, filamentous bacteria, *Streptomyces* species, α-amylase, β-amylase, proteases, submerged fermentation

1. Introduction

Of the estimated worldwide sales of $1.8 billion of the industrial enzymes in 2002, over 45% was accounted for by consumption in the food and feed industry (Shetty *et al*., 2006).

The success of several processes has been due to the availability of enzymes with good activity and stability. In some cases, the operational stability of the enzymes was improved by means of immobilization.

The enzymes from thermopile and hyper thermopile micro organisms that are naturally thermo stable by way of adaptation to the high temperature environments was another focus. With the discovery of new enzymes, the temperature limits of the enzyme activity and stability reached unbelievable extremes.

In contrast to this, the interest in "cold activity" of enzymes is relatively recent, and has been promoted as a result of intense research activity on cold adapted organisms isolated from low temperature environments (Hatti-Kaul *et al*., 2006). One such attempt has been a rather large project sponsored by the EU Fourth Framework research program during the 1990s to examine enzymes from micro organisms isolated in Antarctica, both with respect to fundamental studies on cold adaptation and biotechnological applications (Thomas and Dieckmann, 2002).

Simultaneously, laboratory evolution techniques have provided an alternative and powerful strategy to modify existing enzymes to function at lower temperatures.

A number of reviews on cold active enzymes and mechanisms enabling low temperature activity have appeared in recent years. The applications of enzymes for low temperature processing may provide various benefits.

These include:
- lower energy requirements;
- protection of substrates or products from degradation;
- minimization of side reactions;
- prevention of the destruction of other substances associated with raw material;
- reduced risk of microbial spoilage;

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– inactivation of the enzyme after completion of the process by a mild increase in temperature.

The traditional means of obtaining cold active enzymes has been through screening of naturally occurring cold adapted prokaryotic and eukaryotic organisms for enzymatic activities of interest (Brenchley, 1996; Margesin and Schinner, 1999).

Considering that a major part of Earth, including the vast extent of permanently cold environments such as the Antarctic, Arctic, and mountain regions, as well as the deep sea waters covering three quarters of the planet surface, is exposed to low temperatures (i.e., below 5°C) for a large part of the year, organisms inhabiting such environments should represent a significant portion of the living world. (Price, 2000).

A major challenge for such organisms is to counteract the reduction in chemical reactions due to low temperatures, and the best strategy to maintain sustainable activity under such conditions is to produce cold adapted proteins. Enzymes with up to tenfold higher specific activity are synthesized by psychrophiles to compensate for the low reaction rates. The specific activity is, however; generally lower than that of the mesophilic enzymes at 37°C. Furthermore, the temperature for apparent maximal activity is shifted toward the low temperatures, reflecting the weak stability of these proteins that are prone to unfolding and inactivation at moderate temperatures (Feller, 2003).

Application of cold active enzymes places demand on their cost effective production. It may be stated that cold active enzymes, even though having existed as long as the existence of life in cold environments, have been "discovered" relatively recently. Their potential is yet to be fully exploited. It is very likely that evolution in the laboratory will be used to assist the natural evolution or even gain preference in the design of cold active enzymes with required stability for the applications of interest.

Although protease and amylase are mainly fungal and eubacterial products, the possibility of using *Streptomyces* sp. for enzyme production has recently been investigated. *Streptomyces* species that produce amylases and proteases include: S. clavurigerus, S. *griseus, S. moderatus, S. rimosus, S.thermoviolaceus,S.thermovulgaris, S.aureofaciens* and *S. rimosus* (Yang and Wang, 1999).

The high level of enzyme production using agro-industrial by-products is commercially significant due to cheap nature of these sources. The findings are quite attractive, as only few actinomycetes, particularly have so far been explored for their enzymatic potential and regulation of enzyme synthesis (Metha *et al*., 2006).

This study provides preliminary research regarding the biosynthesis and utilisation conditions of some amylases and proteases crude enzyme extracts obtained with two *Streptomyces* strains, isolated from polar and Romanian soil.

2. Materials and methods

2.1. Micro organisms, media and cultural conditions

Two selected strain of filamentous bacteria used throughout this investigation were *Streptomyces* MIUG 4.116 from Galati University Collection of Micro organisms (acronym MIUG) and a new strain, coded 11P, isolated from polar soil-East Antarctica, the Lassermannn Hills Progress Station region.

The cultivation was carried out in Erlenmeyer flakes, on rotary shaker at 230 rpm and temperature 28ºC, during 8 or 9 days. The medium had the following composition (g/L): soluble starch – 20; corn steep liquor – 10; $(NH_4)_2SO_4$ - 6; $CaCO_3$ - 8; NaCl - 5 and soybean oil - 0,2mL; *p*H = 7,0.

To eliminate other influencing factors use was made of the vegetative inoculum obtained by pure culture cell transfer in a liquid nutrient broth after cultivation to 24-28 hrs on Gauze agar medium (Bahrim, 2003). After 24 hours' cultivation in submerged conditions, 5% cell suspension was used to inoculate the fermentative medium.

2.2. Evaluation of the hydrolase's complex activities

The following enzymatic activities were examined in the liquid culture, after biomass separation at 9000 rot/min, for 10 minutes (Bahrim and Nicolau, 2002a):

– α-***amylase activity*** by using an adapted method (MIUG method) based to a selective distinction of the hydrolysis products in 0.1 N Lugol solution. One α-amylase unit according to this method stands for the amount of enzyme which generates a 0.05 decrease in the optical density, for 1 minute, as measured OD at 610 nm, of the colored iodine-starch colored complex, into a 1% starch solution, at pH 7.0 and 40ºC;

– β-***amylase activity*** by Merck method. One β-amylase unit represents the amount of maltose (in mg) release from 1% starch as substrate, by 1 ml enzymatic liquid at 40ºC, *p*H = 7.0, for one minute;

– ***protease activity*** by Anson modified method. One Anson unit stands for the amount of enzyme which, under the analytical specified conditions (2 % casein as substrate, *p*H = 7.0; for 15 min, at 40°C) hydrolyzes the casein at a speed that facilitates release, in one minute, the hydrolysis products soluble in the trichloroacetic acid; this provides coloration equivalent, measured by spectrophotometry at OD 570 nm, to that of one mole of tyrosine, in the presence of the Folin-Ciocâlteau reagent.

3. Results and discussion

3.1. Enzymes production in submerged cultivation

For the two *Streptomyces* selected strains it had been preserved constant conditions of cultivation in submerse system (on rotary shaker at 230 rpm, temperature 28ºC and time of cultivation 8 days).

In the liquid culture, after biomass separation the following enzymatic activities were examined: α-amylase, β-amylase and protease.

The results are illustrated in figure 1.

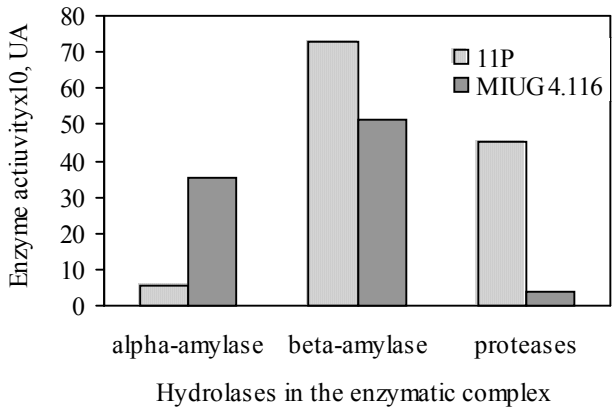

Figure 1. Hydrolase's biosynthesis by selected streptomycetes in submerged cultivation

The data certifies the potential of the selected strains to produce amylases and proteases, the enzyme percentage in crude enzymatic extract depending by strain.

The strain codified 11P wich was isolated from the polar soils is a remarcable β-amylase and protease poducer. These enzymes are produced with high efficiency as compared with *Streptomyces* MUIG 4.116 strain wich was isolated from Romanian soil.

The streptomycetes capability to produce amylases and proteases was highlighted through the evaluation of the enzymes presence in the cultural liquids, obtained through submerse cultivation on rotary shaker at 230 rpm, during 11 days (264 h) at 28°C.

The results are showed in figures 2, 3 and 4.

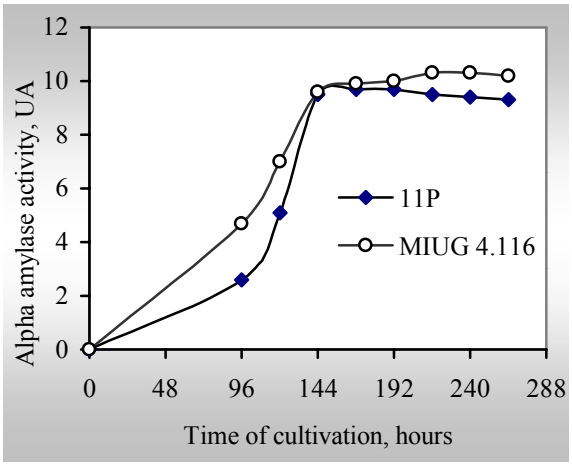

Figure 2. Dynamics of extra cellular alpha-amylase biosynthesis during submerged cultivation of streptomycetes

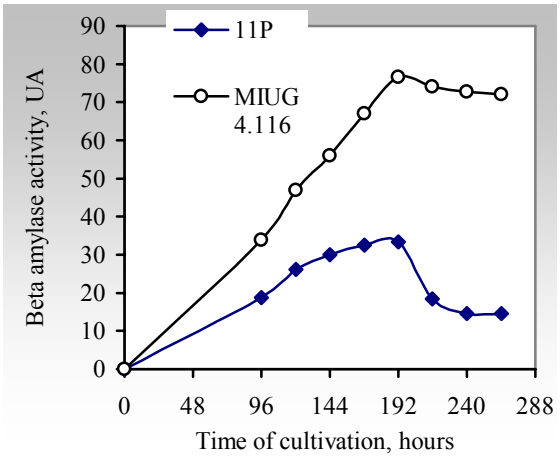

Figure 3. Dynamics of extra cellular beta-amylase biosynthesis by selected streptomycetes

From figure 2 it can be seen that the α-amylase activity at *Streptomyces* MIUG 11P strain is maximum after 192 h (8 days) of submerse cultivation comparing with *Streptomyces* MIUG 4.

116 strain isolated from local soil the α-amylase where the activity is optimal after 216 h (9 days) of submerged fermentation.

At both strains it can be obseved that the α-amylase activity is relative stabile after reaching the biosynthesis maximum efficiency.

The beta-amylase accumulation its optimum after 192 h (8 days) of submerse cultivation at the *Streptomyces* strain codified 11P, isolated from polar soil and also to *Streptomyces* strain MIUG 4.116.

Figure 3 shows that the beta-amilase activity on strain MUIG 4.116 remains relatively constant, instead at the strain 11 P the enzymes accumulation knows an decline maybe as a result of enzyme inactivation or maybe as a rezult of protease denaturante action from the crude extracts.

The proteolitic activity on both strains is optimal after 168 h of cultivation, then the protease activity remain constant (figure 4).

Proteases biosyntesis is previoss to amylases accumulatioon.

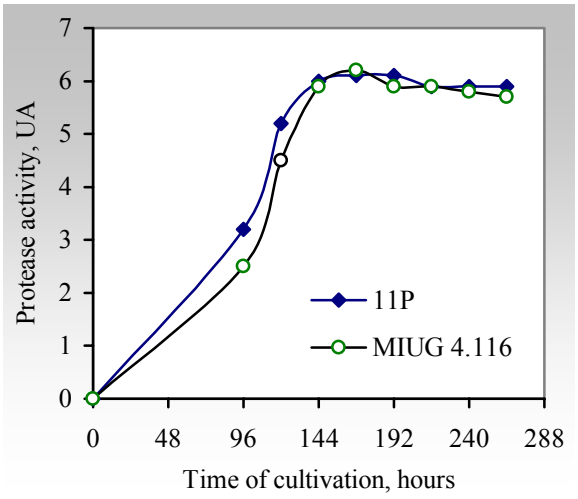

Figure 4. Time course of protease activity of selected *Streptomyces* species in submerged cultivation

The results data certify the potential of the selected strains to produce hydrolytic enzymes (amylase and protease) and encurage future reseach.

3.2. Effect of temperature and *p*H on enzymes activity

For a better efficiency of the hydrolases practical utilization the catalytic properties on diferent conditions on temperature and *p*H were studied. Enzimatic activity for the interval 20…70˚C and also for the pH area 4.0 – 9.0, was evaluated, another parameters remaining constant.

Effect of temperature on amylase and protease activities is presented in figures 5, 6 and 7.

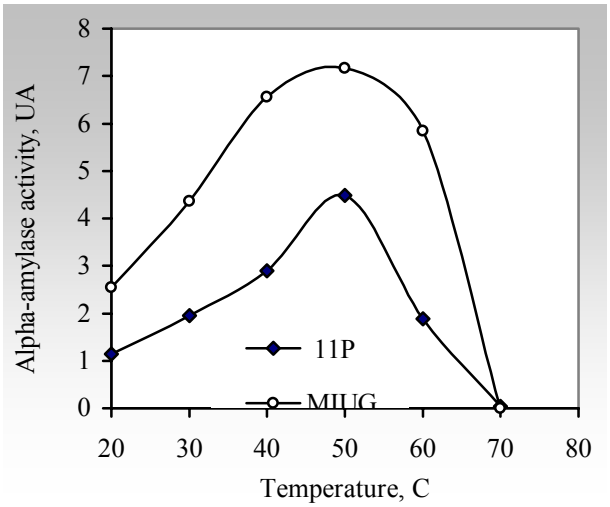

Figure 5. Effect of temperature on α-amylase activity

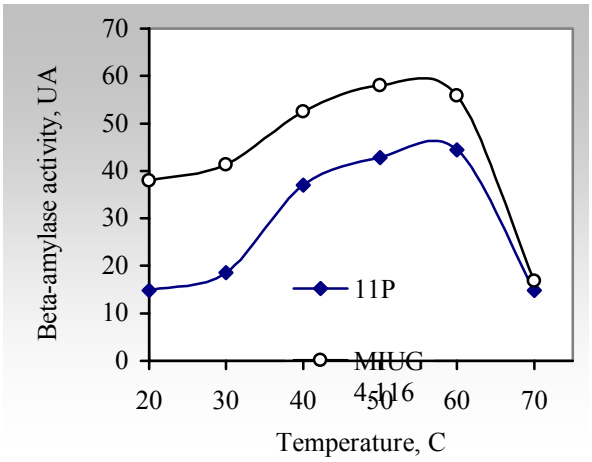

Figure 6. Correlation between temperature and β-amylase activity of streptomycetes

For diagram 5 it can be seen that the α-amylase biosyntetised on both strains have the optimal activity temperature on 50°C.

At temperatures higher than 60°C α-amylase features a drastic inactivation. At 70°C the enzymatic activity is reduced with 2.38 as compared with the maximum value registered at 50°C.

This characteristic makes the distinguish between the α-amylase produced by *Streptomyces* species and

eubacterium, especialy *Bacillus* species that is able to produce α-amylase with high termostability.

The enzyme β-amylase biosyntetised by the strain *Streptomyces* 11P have the optimal activity at 60°C, while the enzyme produced by the MIUG 4.116 strain has the optimal activity at 50°C (figure 6).

At temperatures higher than 60°C beta-amylase activity is poorer at the temperate soil strain, also in the case of the strain isolated from polar soil.

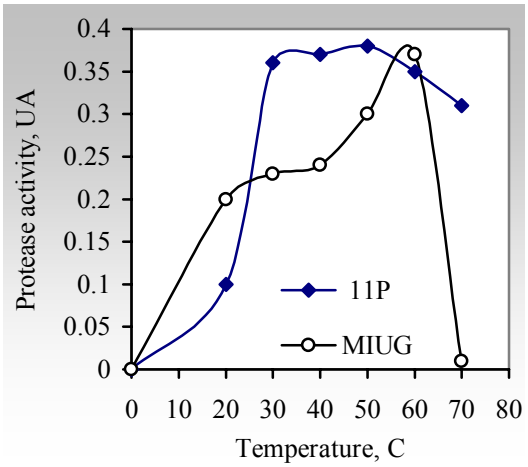

Figure 7. The influence of temperature on protease activity

From the experimental data showed in diagram 7 rezults that the protease produced by the *Steptomyces* 11P wich is isolated from polar soil from Antarctica has the optimal activity at 50˚C, while the protease biosynthesized by *Streptomyces* codified MIUG 4.116 has the optimal activity at the level of 60˚C. In the case of the strain from polar soils it can be observed that after reaching the optimal temperature (60˚C) protease activity registered a drastic decline.

As a conclusion, all 3 enzymes have the optimal activity at temperature within the interval 50…60°C.

Another conclusion is the one that the enzymes from the hydrolyse complex (amylase-protease) are losing form their enzymatic properties when the temperature is higher than 60°C.

The effect of *p*H on amylases activity was measured in *p*H 3.82, 4.85, 5.88, 6.97, 7.87 and 8.74 in 0.2 M citrate and phosphate buffer, at optimum temperature for each enzyme.

The results are showed in figures 8 and 9.

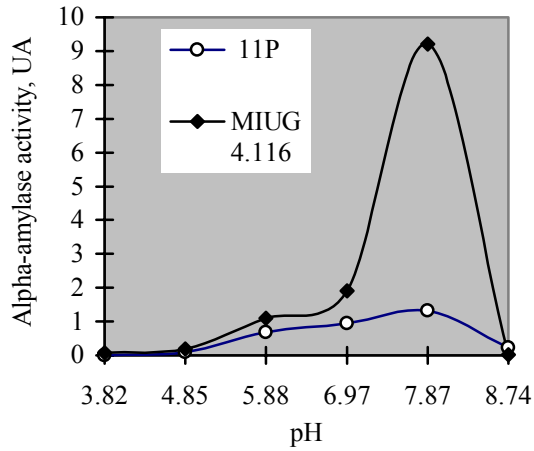

Figure 8. Effect of *p*H on α-amylase activity

Studies proves that the optimal *p*H area for α-amylase activity on both strains is situated within the interval 7.0-8.0 (optimum at *p*H = 7.87).

The α-amylase produced by the *Streptomyces* species is restrained on acid *p*H (*p*H = 3.0-6.0), but also in the alkaline *p*H > 8.0.

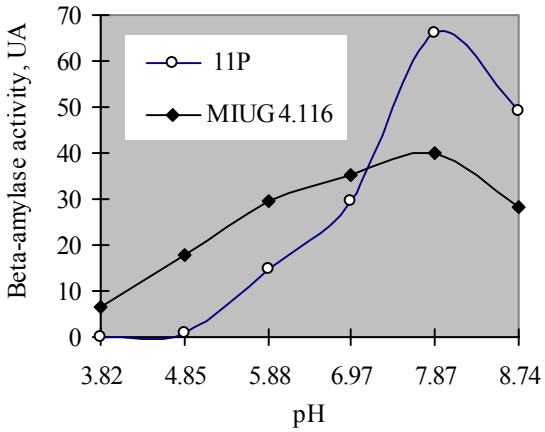

Figure 9. The *p*H influence on *Streptomyces* species β-amylase activity

Figure 9 shows that the β-amylase is optimal activates at alkaline *p*H (*p*H = 7.87) on both studied strains. Data showed that the β-amylase synthesised by *Streptomyces* 11P doesn't action on *p*H value lesser than 5.88. At higher *p*H value than the optimum values of activity, β-amylase remains stable in the field of alkaline *p*H. Beta-amylase produced by the *Streptomyces* MIUG 4.116 strain has moderate activity also at acid pH, but optimum activity is on *p*H=7.87.

Protease biosintetizated by the selected streptomycetes has peak activity on alkaline *p*H.

Enzymes produced by selected strains have maximum activity at *p*H = 8.67 (strain 11P) or *p*H = 7.98 (strain MIUG 4.116) (figure 10).

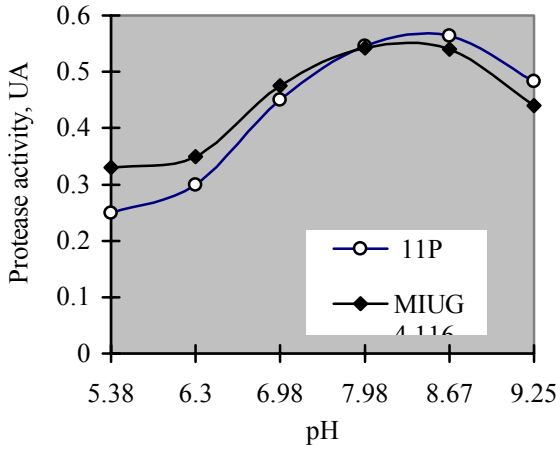

Figure 10. Effect of *p*H on protease activity

4. Conclusions

Study had to prove biotehnologycal conditions evaluation for obtaing and using of crude enzyme complex with hydrolase activity (amylase-protease) biosyntetised by the selected strains of filamentous bacteria.

Streptomyces species are remarcable amylases and proteases producers, the biosyntesis potential depending on the strain, type of enzyme and fermentative conditions.

It has been demostrated that the optimal period of cultivation for obtaing maximum α and β-amylase or proteases biosynthesises is arround 8-9 days.

Amylases produced by the streptomycetes are optimum active at temperatures within 50…60°C and alkaline *p*H (*p*H = 7.87), these properties are different from enzyme characteristics produced by the eubacteria (genus *Bacillus*).

It has been detected small differences between optimal activity conditions specific to proteases produced by the temperate and polar soils filamentous bacteria. The optimum activities are noticed in both cases on 50…60°C and alkaline *p*H.

Acknowledgements

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