G&BM
Tome IV
Iaşi, 2003

ASPECTS CONCERNING THE ENZYMATIC ACTIVITY IN SEVERAL THERMOACTINOMYCETE STRAINS

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Key words: thermoactinomycet, amylase, protease, cellulase, activity, screening, stationary, stirred.

Abstract: In the thermoactinomycete strains subjected to examination the values of their recorded enzymatic activities (i.e. α-amylase, protease, exo-β-1,4 – glucanase, endo -β-1,4 – glucanase and β-glucosidase) were lower in the stationary cultures as compared to the stirred ones. The strain *Thermomonospora fusca* BB_{255} was found to be highly cellulase- producing and at the same time able to synthesize α-amylases and proteases.

INTRODUCTION

The enzymatic equipment of the thermophilic actinomycetes has been extensively studied by numerous researchers; as a result one can find many information on this matter.

The thermoactinomycetes, and particularly the strains belonging to the genera *Thermoactinomyces* and *Thermomonospora* have proved highly enzyme-producing: they produce amylases, proteases, cellulases, lipases, etc.

The α-amylases produced by thermoactinomycetes exhibit their activity at high temperatures; this is the reason why they raise many specialists' interest (Heese, O., Hansen, G., Hohne, W.E., Korner, D., 1991).

The research studies performed have shown that this group of microorganisms is an inexhaustible source of proteolytic enzymes; due to their rich enzymatic equipment they are capable to produce various proteolytic enzymes, some of them active on substrates quite resistant to degradation by other microorganisms (Trigo, C., Ball, A.S., 1994).

Many studies have been carried out on the species *Thermomonospora curvata* to determine its nutritional needs in a chemically defined environment and to compare its capacity to degrade various natural and artificial cellulose materials (Beguin, P., Aubert, J.P., 1994). Such studies are expected to provide the basis for handling the controlled composting conditions in order to increase the rate and the efficiency of cellulose degradation.

THE AIM OF INVESTIGATIONS

Our investigations focused on the selection of the best α-amylases, proteases, and cellulases producing strains of thermoactinomycetes.

MATERIAL AND METHOD

With a view to a rigorous testing of the active thermoactinomycetes strains the production of α-amylases, proteases, and cellulases was tested successively, in conformity with the methodology recommended by the literature:

- qualitative screening – by assessing colony growth;
- semi-quantitative screening – by measuring starch hydrolysis, casein lysis and cellulose lysis zones;
- quantitative screening – by dosing the α-amylase, protease, and cellulase activities.

Qualitative screening

The first selection of the isolated strains was based on their capacity to use starch, casein and respectively cellulose when grown on media containing 2% of starch (as a sole carbon source), 1% of casein (as a sole nitrogen source) and 1% of cellulose (as a sole carbon source).

The isolated microorganisms were grown on selective media as follows:

- medium Kuster and Williams (1964) for amylases;
- medium Waksman (1961) for proteases;
- medium Lechevalier (1963) for cellulases.

Following cultivation on the specified media, in Petri dishes, at 55^0C for 5 to 7 days, the strains which exhibited no growth were eliminated.

Semi-quantitative screening

At this stage the selection was performed based on the ratio of the diameters of the starch hydrolysis zone – Dza – (revealed using Lugol solution 0.1N), casein lysis zone – Dzc – and respectively cellulose lysis zone – Dz cel – (revealed by culture media clarifying) to the diameter of the colony (Dc) formed by cultivation on selective media, determined by keeping the plates in a thermostat at 55^0C for 5 to 7 days.

Quantitative screening

In order to find the best producing strains of α-amylases, proteases, and cellulases, those found most active following the semi-quantitative screening performed were subjected to submerged cultivation in stationary and stirred liquid medium (the same used for the qualitative and semi-quantitative screening), inside a thermostat chamber, at 55^0C for 5 to 7 days.

The methods used to assay α-amylase, protease, and cellulase activities are the following:

- α-amylase activity: Noelting and Bernfeld method (quoted by Artenie an Tanase, 1981);
- protease activity: Anson method (qtd. by Dumitru and Iordanescu, 1981);
- cellulase activity: dosing of exo-β-1,4 – glucanase, endo -β-1,4 – glucanase and β- glucosidase activities – Peterson method (qtd. by Dumitru and Iordanescu, 1981).

Statistic calculus – the experimental data obtained were statistically processed according to the Student test (Morait,Gh., Roman, I.,1983; Muresan, P., 1989).

RESULTS AND DISCUSSIONS

A number of 71 thermophilic actinomycetes strains were tested. The strains were isolated (as pure cultures) from various sources, such as: compost for mushroom growing (at various stages of preparation), fermented manure of various origins (cattle, horses, sheep and swine), moldy hay, straws, soil (cultivated and uncultivated), and peat.

Upon qualitative and semi-quantitative screening of the amylolitic activity, a number of 21 strains of thermoactinomycetes were retained to determine the dynamics of their α-amylase activity (24, 48, 72, 96 and 120 hours) in submerged stationary and stirred cultures. The results showed variations of the α-amylase activity not only from one strain to another but also at the same strain, depending on the growth conditions. Thus, at most of the strains the α-amylase activity peaked after 96 h of cultivation, both in stationary and in stirred conditions, except for 4 strains (*Actinomadura flexuosa* SN_{555}, *Saccharomonospora viridis* SC_{955}, *Thermoactinomyces thalpophilus* C_{5955} and

Thermomonospora curvata SC_{655}) at which the maximum α-amylase activity was reached after 72 of cultivation under the same conditions. The values of the α-amylase activity vary from one strain to another between 0.005 – 10.221 μM maltose/ml at the stationary cultures and 0.010 – 12.724 μM maltose/ml at the stirred ones, which correspond to the specific activities of 4.000 – 20.239 μM maltose/mg protein and 5.000 – 21.206 μM maltose/mg protein respectively. Two very highly α-amylase producing strains were selected: *Thermoactinomyces vulgaris* F_{2455} and *Thermoactinomyces thalpophilus* P_{1055} which exhibited an α-amylase activity of 9.387 μM maltose/ml and respectively 12.724 μM maltose/ml (9.52% of the tested strains).

The comparative examination of the strain *Thermoactinomyces thalpophilus* P_{1055} and the reference strain (i.e. *Thermoactinomyces vulgaris* F_{2455}) showed that the differences in the α-amylase activity are insignificant ($p>0.1$) when the strains are cultivated under stationary conditions; however, under stirred conditions, the differences are significant ($p<0.01$)- Fig.1

By qualitative and semi-quantitative screening of the proteolytic activity, from 71 strains of thermoactinomycetes a number of 10 were retained to determine the dynamics of their proteolytic activity (24, 48, 72, 96 and 120 hours) in submerged stationary and stirred cultures.

The results obtained showed that most of the strains reach the maximum of proteolytic activity 96 h after inoculation, both in stationary and in stirred conditions, except for the strains *Thermoactinomyces sacchari* C_{1555} (proteolytic activity peaked after 48 h of cultivation in stirred cultures, equivalent to a specific activity of 0.919 U/mg protein) and *Thermomonospora chromogena* P_{555} (proteolytic activity peaked after 72 h of cultivation in stirred cultures, equivalent to a specific activity of 0.415 U/mg protein).

The values of the proteolytic activity vary from one strain to another between 0.077 – 5.295 U/ml at the stirred cultures and 0.038 – 2.375 U/ml at the

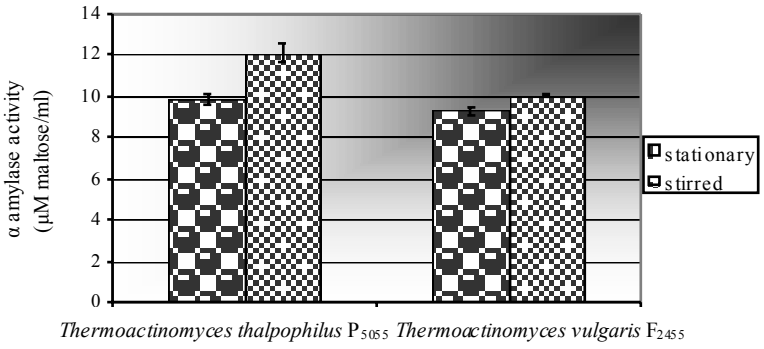

Fig. 1 - Variation of α-amylase activity in the selected strains (μM maltose/ml)

stationary ones, which correspond to the specific activities of 0.155 – 8.929 U/mg protein and 0.045 – 2.714 U/mg protein, respectively.

Three very highly protease producing strains were selected: *Faenia rectivirgula* F_{755}, *Thermoactinomyces vulgaris* F_{2455} and *Thermomonospora alba* C_{4255} which exhibited a proteolytic activity between 2.375 and 5.295 U/ml (30% of the tested strains).

The comparative examination of the strains *Thermomonospora alba* C_{4255}, *Faenia rectivirgula* F_{755} and the reference strain (i.e. *Thermoactinomyces vulgaris* F_{2455}) showed that under stationary conditions the differences in the proteolytic activity are insignificant ($p>0.1$) in the first case and significant ($p<0.01$) in the second one. Under stirred conditions both strains exhibited higher proteolytic activities as compared to the reference strain, the differences being significant ($p<0.01$).The results of the comparative analysis of each strain's proteolytic activity under stationary and stirred conditions showed that the differences are significant ($p<0.001$)- Fig.2

The results obtained by analysing the dynamics of celullase activity (24, 48, 72, 96 and 120 hours) in 9 thermoactinomycete strains (selected by qualitative and semi-quantitative screening) tested in submerged cultures showed that most strains (77.77%) exhibited maximum exo-β-1,4 – glucanase, endo -β-1,4 – glucanase and β- glucosidase activities 144 h after inoculation, both under stationary and stirred conditions. However, 2 strains, *Pseudonocardia thermophila* BB_{2555} and *Pseudonocardia thermophila* C_{7355}, exhibited a maximum activity 120 h after inoculation, under both growth conditions.

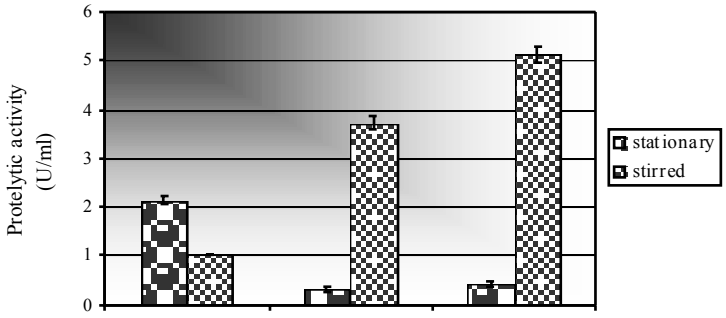

Fig. 2 - Variation of protease activity in the selected strains (U/ml)

Not only under stationary conditions but also under stirred ones, the biosynthesis of endo -β-1,4 – glucanase was very slow during the first 96 h but increased significantly afterwards until it reached the peak value.

The monitoring of the same aspect for exo-β-1,4 – glucanase showed that 33.33% of the strains exhibit a slow increase of the activity up to 120 h after inoculation, reaching the peak activity 114 h after inoculation while in the strains *Pseudonocardia thermophila* BB_{2555} and *Pseudonocardia thermophila* C_{7355} the biosynthesis of the

enzyme is slow up to 96 h reaching peak activity 120 h after inoculation, both under stationary and stirred conditions.

In what concerns the β- glucosidase activity, the strains tested both under stationary and under stirred conditions were found to have a relatively uniform behavior. Thus, β- glucosidase activity in the strains *Pseudonocardia thermophila* BB_{2555} and *Pseudonocardia thermophila* C_{7355} peaked 120 h after inoculation while in the rest it peaked at 144 h of cultivation and then decreased at 168 h.

The values of the exo-β-1,4 – glucanase activity range between 0.046 and 0.047 U/ml/min under stationary conditions and 0.061 and 0.520 U/ml/min under stirred conditions.

The activity of endo -β-1,4 – glucanase varied between 0.154 U/ml/min and 5.633 U/ml/min under stationary conditions and between 0.170 U/ml/min and 7.552 U/ml/min under stirred conditions.

The β- glucosidase (cellobiase) activity varied between 0.011 and 0.085 U/ml/min under stationary conditions and between 0.023 and 0.348 U/ml/min under stirred conditions.

The general analysis of the examined cellulase activities showed that the highest values were obtained for endo -β-1,4 – glucanase followed by exo-β-1,4 – glucanase and β- glucosidase. Based on the values of the cellulase activities obtained from the 9 thermoactinomycete strains tested by submerged cultivation, a number of 2 strains were selected as highly cellulase productive: *Thermomonospora curvata* SC_{655} and *Thermomonospora fusca* BB_{255} (22.22% of the strains subjected to examination).

The comparative examination of the strains *Thermomonospora fusca* BB_{255} and the reference strain (i.e. *Thermomonospora curvata* SC_{655}) showed that under stationary cultivation conditions the differences in the exo-β-1,4 – glucanase activity are not very significant ($p<0.05$), in endo -β-1,4 – glucanase activity are significant ($p<0.001$) and in β- glucosidase activity are significant ($p>0.05$) while under stirred cultivation conditions the differences are significant for all the three examined enzymes (Fig.3,Fig.4,Fig.5).

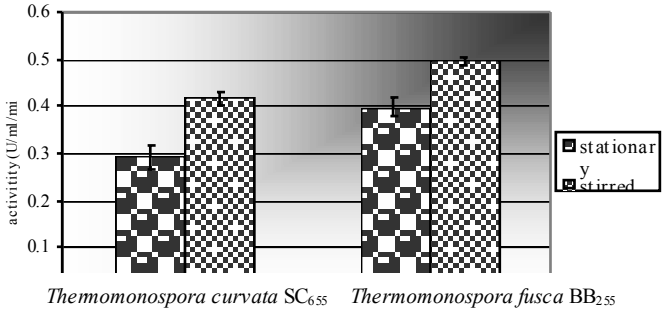

Fig. 3 - Variation of exo-β-1,4 – glucanase activity in the selected strains (U/ml/min)

CONCLUSIONS

The values of the enzymatic activities (i.e. α-amylase, protease, exo-β-1,4 – glucanase, endo -β-1,4 – glucanase and β- glucosidase activities) in the stationary cultures are lower as compared to those recorded in the stirred cultures. This may be due to the fact that under stationary conditions, the quantity of dissolved O_2 in the culture broth is smaller and inhibits aerobe thermoactinomycetes.

Highly α-amylase producing strains (*Thermoactinomyces vulgaris* F_{2455} and *Thermoactinomyces thalpophilus* P_{1055}) also synthesize proteases, but are not cellulase-producing.

Highly protease-producing strains (*Faenia rectivirgula* F_{755} and *Thermoactinomyces vulgaris* F_{2455}) also synthesize α-amylases but cellulases, except for the strain *Thermomonospora alba* C_{4255} with a good cellulase synthesis potential.

Of the highest cellulase producing strains, *Thermomonospora fusca* BB_{255} synthesizes both α-amylases and proteases, while the strain *Thermomonospora curvata* SC_{655} has a low α-amylase producing potential and lacks the capacity to synthesize proteases.

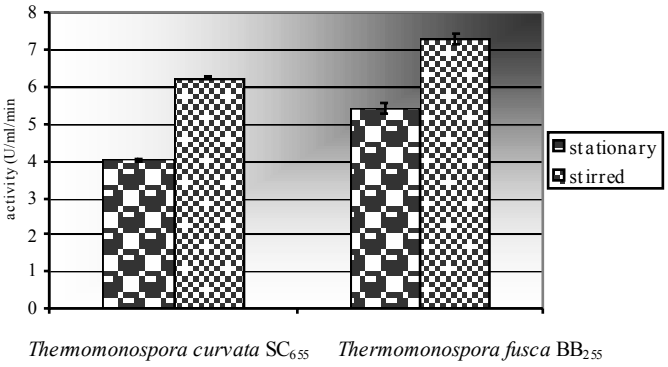

Fig. 4 - Variation of endo -β -1,4 – glucanase activity in the selected strains (U/ml/min)

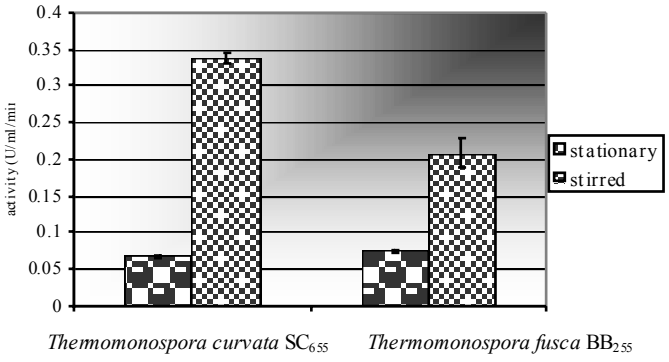

Fig. 5 - Variation of β - glucosidase activity in the selected strains (U/ml/min)

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