COLD-ADAPTATION AND ALKALINE HYDROLYTIC PROPRIETIES OF THE POLAR STREPTOMYCETES PREDICTION ON PLATE ASSAY, BASED ON INSOLUBLE CHROMOGENIC SUBSTRATES WITH AZURINE CROSS-LINKED

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Abstract
A semi-qualitative screening based on protease and amylase activity evaluation in a basal agar medium supplemented with insoluble chromogenic substrates based on AZCL (Azurine-Crosslinked with amylose or casein) using a plate assay was used for selecting the polar streptomycetes able to produce cold actives and alkaline amylases and proteases. This technique provides a specific and rapid simultaneous detection of high active hydrolase producing strains based on the visible solubilization of small particles of AZCL and the formation of haloes on plates. It has a great potential in increasing the efficacy of screening streptomycetes able to produce hydrolytic enzymes. This study revealed the potential of the selected streptomycetes isolated from polar soils to biosynthesize amylases and proteases cold-adapted at low temperatures (from 5 to 20ºC) and alkaline pH values (8 to 9).

Keywords: *Streptomyces sp*., polar strains, proteases, amylases, cold-adapted enzymes, plate assay screening, insoluble chromogenic substrates, Azurine-cross-linked (AZCL)

1. Introduction

Extremophilic microorganisms are adapted to live at high temperatures in volcanic springs, at low temperatures in the cold polar regions, at high pressure in the deep sea, at very low or high *p*H values (*p*H 0-3.0 or *p*H 10.0-12.0), or at very high salt concentrations (5% - 30%) (Hoyoux, 2004).

Cold-adapted organisms, psychrophiles and psychrotrophs, inhabit both terrestrial and aquatic environments in polar and alpine regions, in the bulk of the ocean, in shallow subterranean regions, in the upper atmosphere, in refrigerated environments, and on plants and animals living in cold regions. Psychrotolerant organisms grow well at temperatures close to the freezing point of water, but have the fastest growth rates above 20ºC, whereas psychrophilic organisms grow faster at a temperature of 15ºC or lower, but are unable to grow above 20ºC (Madiga, 2005).

Owing to the fact that cold completely permeates micro organisms, all components of the cell, from membranes and transport system to intracellular solutes, nucleic acids and proteins, must be suitably adapted. Moreover, fundamental cellular processes of metabolism, replication, transcription and translation must also be adapted to withstand to cold. Translation appears to be the most sensitive process to the temperatures; associated proteins play an important role in temperature sensing. At low temperatures, the low kinetic energy of reacting molecules is compensated for by the flexible structures of cold-active enzymes. Flexibility is achieved by combining different structural features, which may include the hydrophobic core reduction, decreased ionic and electrostatic interactions, and increased charge of surface residues that promote increased solvent interaction, additional surface loops, and substitution of proline residues by

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glycines in surface loops, a decreased arginine/lysine ratio, less interdomain and subunit interactions and fewer aromatic interactions.
Therefore the active site and the adjacent regions of cold-active enzymes remain flexible, and the increased conformational flexibility is accompanied by increased thermolability.

The high flexibility of cold-active enzymes enables increased complementarity between the active site and substrate, at a low energy cost, resulting in high specific activity at low temperatures (Park, 2006, Rajni, 2006). The two properties of cold-active enzymes with the most obvious biotechnological application are their high catalytic activity at low temperatures and low termostability at elevated temperatures.

The catalytic properties (temperature optima, half-lives, melting temperatures, $k_{cat,}$ V_{max}, k_m and activation energies) of numerous enzymes isolated from cold-adapted organisms are available (Hoyoux, 2004; Georlette, 2004; Cavicchioli, 2002).
Cold-active enzymes have important industrial applications in food industry, as well as in biomass conversion, bioremediation, since the processes run at low temperatures reducing the risks of contamination by mesophiles and also saving energy (Rob, 1992; Metha, 2006).

The plate screening methods with insoluble chromogenic substrates, as AZCL coupled by polymeric substrates, provide an array of relatively straightforward and simply applicable tools for specific detection of polymer-degrading cold-active enzymes (Margesin, 1999; Ten, 2004).

AZCL-Polymers are prepared by dyeing and crosslinking highly purified polymers (amylose, cellulose, xylan, chitosan, casein etc). They are supplied as a fine powder (milled to pass a 0.5 mm screen). These substrates are insoluble in buffered solutions, but rapidly hydrate to form gel particles which are readily and rapidly hydrolysed by specific endo-hydrolases releasing soluble dye-labelled fragments. These substrates can be used to locate enzyme activities in electrophoresis gels and to locate specific enzyme producing micro organisms on culture plates.

The aim of this study was to develop new plate techniques for semi-quantitative screening of polar streptomycetes able to biosynthesis cold-adapted and alkaline amylases and proteases.

2. Material and Methods

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

Microorganisms: The *Streptomyces* strains were isolated from Antarctic soil sampled from East Antarctica coast. Were studied 30 *Streptomyces* strains, 7 strains from "Dunarea de Jos" University Micro organisms Collection (coded MIUG) and 23 new isolated, from polar soils from Grove Mountains and Progress 2 Lake, in 2004.

Screening media and procedure

Insoluble chromogenic substrates agar plate media: A basal starch-casein medium, containing (g/L): starch - 10.0; casein - 0.3; KNO_3 - 2.0; NaCl - 2.0; $MgSO_47H_2O$ - 0.05; $CaCO_3$ -0.02; $FeSO_47H_2O$- 0.01 and agar -12.0, was supplemented by adding 0.05% of insoluble chromogenic functionalized a substrates based on AZCL. First the commercial AZCL-Amylose and AZCL-Casein powders were transferred into 96% ethanol and then added into the basal medium. To abide the particles dispersed, the autoclaved medium was agitated gently before being poured into plates.

Semi-quantitative plate agar screening procedure

To detect the amylose and casein hydrolyzing streptomycetes which are active at different temperatures, the medium supplemented with insoluble chromogenic substrates was inoculated "in point" with cells and incubated at different temperatures (5ºC, 10ºC, 15ºC, 20ºC, 28ºC and 37ºC). After 10 days, the plates were visually inspected and the growth performance of the individual colonies was interpreted on a scale from – (no growth) to ++ (good growth) based on the size of the blue zone developed around the colonies (Stougaard, 2002). The same technique was used for screening both amylase and casein-degrading streptomycetes adapted at different values of pH, with the difference that the agar medium was adjusted with different buffer solutions. 2 mL buffer solution was pipetted in each plate and the basal medium with insoluble chromogenic substrates was added. The Petri dishes were then incubated at 20ºC.
To detect the catalytic properties of the enzymatic extracts at different temperatures and pH values the

basal medium supplemented with AZCL was adjusted at different *p*H values ranging from 4.0 to 9.0. Volumes of 300 - 350 µL crude enzymatic extracts were placed in the 0.5 cm size wells made into the agar medium. The plates were afterwards incubated at different temperatures.
The crude enzymatic extract was obtained as a result of the biomass separation at 9000 rpm, for 10 minutes, after the submerged cultivation in Erlenmeyer flakes on rotary shaker at 230 rpm, at 20ºC, for 10 days on the liquid medium with the following composition (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 and *p*H = 7.0 (Bahrim, 2002).

3. Results and Discussion

The potential of biosynthesizing cold adapted amylases and proteases was estimated by testing the ability of the polar streptomycetes strains to metabolise insoluble chromogenic substrates based on azurine-crosslinked with amylose or casein (AZCL-Amylose, AZCL-Casein). The bacteria produce extra cellular enzymes which hydrolyse the large substrate insoluble molecules dyed with AZCL. The small hydrolyzed compounds are still dyed blue and diffuse in the plate developing blue circle zones around the colonies (Figure 1).
The chromogenic substrate AZCL-Amylose particles were degraded by all tested streptomycetes strains.
The capacity of tested *Streptomyces* sp. to grow and produce amylases and proteases at different temperatures and pH values was established by plating the strains on basal media supplemented with 0.05% AZCL-Amylose or 0.05% AZCL-Casein, followed by incubation at different temperatures and different pH values.

Most of the studied polar streptomycetes strains are able to grow and biosynthesize (amylases and proteases) at low temperatures ranging between 15°C and 20°C (Figure 2 and Figure 3).
According to Figure 4 and Figure 5 most of the selected streptomycetes strains have the capacity to grow and to biosynthesize amylases and proteases at alkaline pH values.
The results certify the streptomycetes ability to grow and produce amylases and proteases cold adapted and alkaline *p*H active.

The enzymatic potential and catalytic properties of crude extracts after submerged cultivation were also evaluated by adding into the wells of the agar media 300 - 350 µL of enzymatic liquid culture.

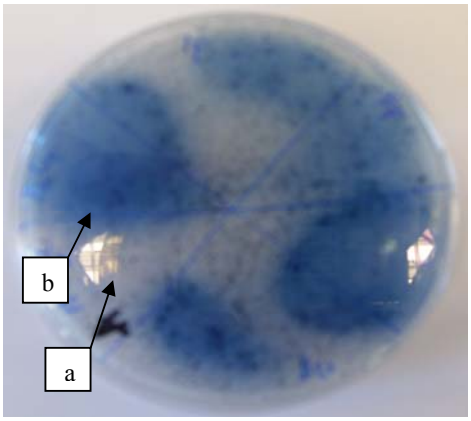

Figure 1. Hydrolytically degrading streptomycetes ability, at 20ºC and pH 7.0, in basal agar media, containing 0.05% insoluble chromogenic substrates based on AZCL; a) medium with insoluble chromogenic substrates, b) the presence of the hydrolysed zones give indication about the fact that the selected *Streptomyces* strains are able to produce hydrolytical enzymes

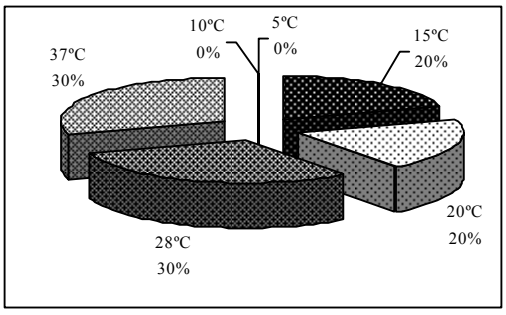

Figure 2. Polar streptomycetes ability to grow and biosynthesize amylases at different temperatures

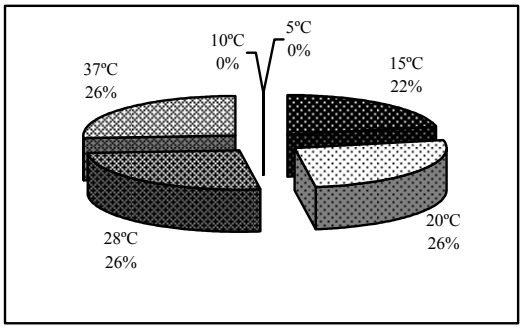

Figure 3. Polar streptomycetes ability to grow and biosynthesize proteases at different temperatures

The plates were incubated at different temperatures and tested at different *p*H values. The results are shown in Figure 6 and Figure 7.
Most of the studied polar streptomycetes are able to produce cold adapted amylases and proteases active at 5-20°C.

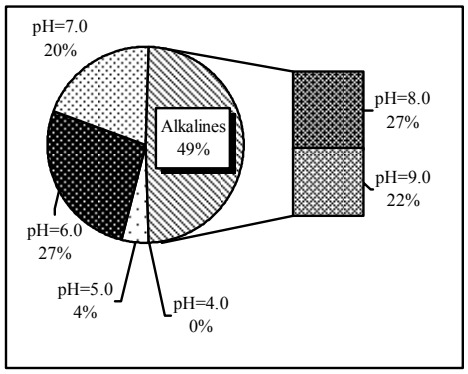

Figure 4. Streptomycetes growth ability and proteases biosynthesis at different *p*H values

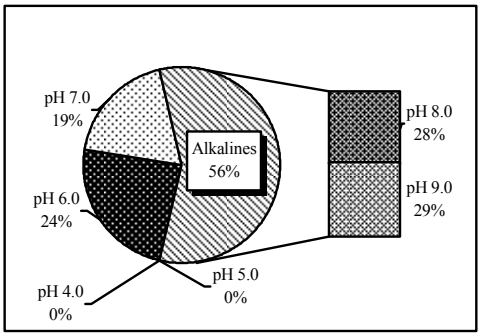

Figure 5. Polar streptomycetes growth ability and amylases biosynthesis at different *p*H values

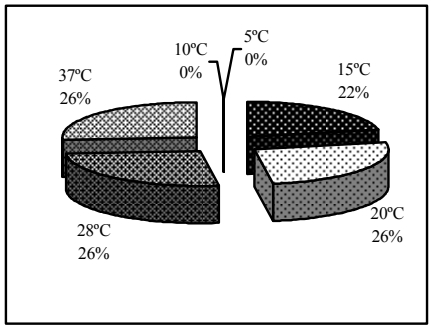

Figure 6. Amylase activity of the tested polar streptomycetes after 24 h incubation on AZCL-Amylose

The strains with high activity and stability at 20°C are the most interesting strains to be studied due to their direct applications in the biotechnological field. The use of psychrophilic enzymes allows energy saving by avoiding expensive heating steps. Moreover, undesirable chemical reactions occurring at high temperatures can be minimised. These enzymes are thermally labile therefore can be easily and rapidly inactivated when required. The rapid inactivation of cold-active enzymes by mild heat treatment preserves product quality, permits selective enzyme inactivation in a complex medium, and also does not require expensive heating/cooling systems. Consequently, the cold-adapted enzymes have numerous applications in biotechnological and industrial fields (Hoyoux, 2004).

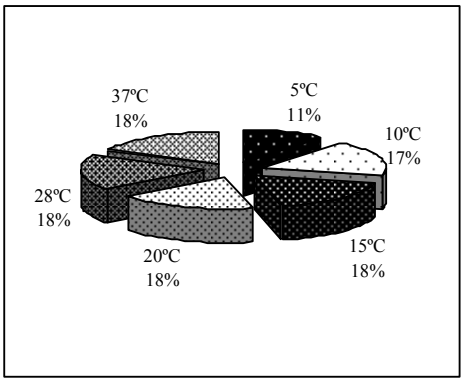

Figure 7. Protease activity of the polar streptomycetes after 24 h incubation on AZCL-Casein

The results certify the streptomycetes ability to produce amylases and proteases cold adapted and alkaline *p*H active (Figure 8, Figure 9 and Figure 10).

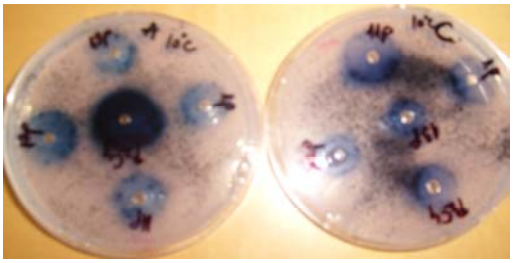

Figure 8. Amylose (left) and casein (right) degrading enzymes produced by selected streptomycetes, at 10ºC and *p*H 7.0, in agar media containing 0.05% AZCL-Amylose and 0.05% AZCL- Casein

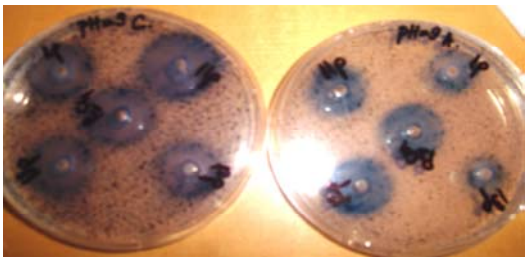

Figure 9. Amylose and casein degrading enzymes active at *p*H 9.0 and 20ºC, in agar media containing 0.05% AZCL-Amylose (right) and 0.05% AZCL- Casein (left)

From figure 8 and figure 9 it can be seen that the hydrolysis zone varied with the temperature and pH values; some strains produced a high level of cold active enzymes.

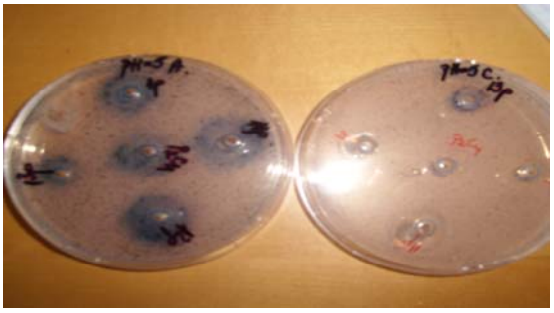

Figure 10. Evaluation of amylose and casein degrading enzyme activity of the selected polar streptomycetes, at *p*H 5.0 and 20ºC, in basal agar medium supplemented with 0.05% AZCL-Amylose (left) and 0.05% AZCL-Casein (right)

That means that the tested proteases biosynthesised by the selected streptomycetes are not active at *p*H 5.0 in comparison with the alkaline values (figure 10 and figure 9) where all strains are able to produce enzymes.

It can also be seen that increasing the *p*H, the *Streptomyces sp* produce more hydrolytic enzymes, the hydrolysis zone at *p*H 9.0 being more extended than the blue zones developed at *p*H 7.0, correlated with the temperatures.

The rate of substrate hydrolysis was also tested by pouring 300-350 µL of enzymatic liquid culture into the wells. After 24 h the substrate hydrolysis potential was evaluated. The plates were incubated at different temperatures and tested at different *p*H values. The strains coded MIUG 1P, MIUG 11P, MIUG 12P and P2C4 show a high hydrolytically-degrading potential after 24 h at temperatures of 5-37ºC.

As a result of the 30 strains studied, the strains that released the biggest performances were *Streptomyces* strains coded MIUG 1P, MIUG 11P, MIUG 12P, MIUG 13P and P2C4. Owing to their growth and excellent production of cold-adapted active enzymes (amylases and proteases) at alkaline *p*H values, these strains will be considered in the following studies as selected agents to produce new cold active and alkaline enzymes with industrial application.

4. Conclusions

This new plate technique provides unique possibility of selecting straight in the primary screening the active enzymes producing microorganisms (Rajni, 2006).
The advantages of this screening technique can be summarized as follows: different polymer-degrading micro organisms can be detected using corresponding chromogenic substrates as medium supplements. The plate assay is simple, rapid and adapted for screening of a large number of strains. The diameter of the halo zone is very useful for predicting the enzyme yield as an aid to select strains with high level of polymer-degrading activities.
The results of this study certify the streptomycetes ability, to produce cold adapted and alkaline *p*H actives amylases and proteases.
Cold adapted and alkaline hydrolytic amylases and proteases biosynthesised by studied polar streptomycetes can be successfully used in bioremediation process, dairy industry, and detergent making.

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