Screening of polar streptomycetes able to produce cold-active hydrolytic enzymes using common and chromogenic substrates

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Abstract

A semi-qualitative screening based by proteases and amylases production on Gauze agar media with 1% starch or 1% casein, as single carbon or nitrogen sources was made first time by using radial diffusion in gel method for active cold adapted strains selection by cultivation from 5ºC to 20ºC. Another step of screening was made on starch or casein medium adjusted with insoluble chromogenic functionalized substrates based on AZCL (azurine-crosslinked amylose or casein) using a plate assay based on the visible solubilization of small particles of AZCL and the formation of blue haloes around the active colonies growth on agar media on Petri dishes. These substrates are insoluble in buffered solutions, but rapidly hydrate to form gel particles which are readily and rapidly hydrolysed by specific hydrolase's releasing soluble dye-labelled fragments by develop a blue specific zone around the colony during cultivation. This technique provides a specific, reliable and rapid simultaneous detection of high active hydrolase's producing strains. It has a great potential for increasing the efficacy of screening streptomycetes able to produce hydrolytic enzymes as amylases and proteases. The selected streptomycetes strains isolated from polar soils are able to biosynthesis amylases and proteases cold-adapted at low temperatures (from 5 to 20ºC) and alkaline pH.

Keywords: *Streptomyces sp*., polar strains, cold-adapted enzymes, plate assay screening, insoluble chromogenic substrates, Azurine-crosslinked, AZCL-Amylose, AZCL-Casein, proteases, amylases

Introduction

Extremophilic micro organisms 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 and high pH values (pH 0-3.0 or pH 10.0-12.0), or at very high salt concentrations (5% - 30%) [2].

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 20 ºC, whereas psychrophilic organisms grow faster at a temperature of 15 ºC or lower, but are unable to grow above 20 ºC [3].

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. Similarly, fundamental cellular processes of metabolism, replication, transcription and translation must also be adapted to withstand to cold. The most sensitive process on temperature appears to be translation and the ribosome, and associated proteins play an important role in temperature sensing. At low temperature, the low kinetic energy of reacting molecules is compensated for by the flexible structures of cold-active enzymes. Flexibility is achieved by a combination of structural features, which may include a reduction in core hydrophobicity, decreased ionic and electrostatic interactions, increased charge of surface residues that promote increased solvent interaction, additional surface loops, and substitution of praline residues by glycines in surface loops, a decreased arginine/lysine ratio, less interdomain and subunit interactions and fewer aromatic interactions. The net effect is that the active site and adjoining regions of cold-active enzymes remain flexible, and the increased conformational flexibility is accompanied by increased thermolability. The high flexibility of cold-active enzymes enable increased complementarily between the active site and substrate, at a low energy cost, resulting in high specific activity at low temperatures, [6,7].

The two properties of cold-active enzymes that have 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 [2].

Cold-active enzymes have important biotechnological applications in food industry, as well as in biomass conversion, bioremediation, inasmuch as running processes at low temperatures reduces the risk of contamination by mesophiles and also saves energy [5,8].

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 polymers -degrading cold-active enzymes [4,10].

AZCL-Polymers (Azurine-Crosslinked 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 amylases and proteases.

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 sampling 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 Spitzbergen Archipelago.

Screening media and procedure

Common substrates agar plates media: The qualitative screening based by proteases and amylases production was made on basal Gauze agar medium containing (g/l): K_2HPO_4 - 0.5; MgSO4 7 H_2O - 0.5; KNO_3 - 1.0, NaCl - 0.5; $FeSO_4$ $2H_2O$ - 0.01 and agar - 25.0, supplemented with 1% starch and 1% casein, as single carbon or nitrogen sources.

Insoluble chromogenic substrates agar plate media: As 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% the insoluble chromogenic functionalized substrates based on AZCL. The commercial AZCL-Amylose and AZCL-Casein powers were transferred in 96% ethanol and added into the basal medium. To abide the particles dispersed, autoclaved medium was agitated gently being poured into plates.

Semi-quantitative plate agar screening procedure: For screening polymers-degrading streptomycetes using classical techniques, the *Streptomyces* sp. cells were inoculated "in point" on surface of agar plate's media containing common substrates. The Petri plates were incubated at different temperatures 5ºC, 10ºC, 15ºC and 20ºC. After 4-10 days of cultivation the hydrolytic potential was evaluated based on substrates hydrolysis zone development as colourless zone on medium with casein and the same effect on medium with starch, after surface washing with 0.1N Lugol solution. A semi-quantitative screening based on Substrate Hydrolysis Index (S.H.I.) evaluation was realized. Substrate Hydrolysis Index (S.H.I.) was calculated as ration between mean diameter of substrate hydrolysis zone (d) and mean diameter of bacterial colony (D), both expressed in millimeters.

To detect polymers-degrading streptomycetes active at low temperatures the medium supplemented with insoluble chromogenic substrates was point inoculated with cells and incubated on different temperatures 5ºC, 10ºC, 15ºC and 20ºC. After 10 days the plates were visually inspected and growth performance of the individual colonies was interpreted on a scale from – (no growth) to ++ (good growth) based of the size of blue zone developed around the colonies [9].

For screening polysaccharide-degrading streptomycetes adapted on different values of pH were used the same technique as was fore mentioned, only that the agar medium was adjusted with different buffer solutions. For pH 6.0, 7.0 and 8.0 were prepared 0.2 M phosphate buffer solutions and also for pH 9.0 was made 0.2 M carbonate-bicarbonate buffer. In each plate were pipettes 2 mL buffer solution and after that was added the basal medium with insoluble chromogenic substrates. The Petri dishes were incubated at 20ºC.

To detect the enzymatic activity at lower temperatures and alkaline pH values the basal medium supplemented with AZCL was adjusted at the different pH values varying from pH 6.0 to pH 9.0. After the medium solidification some wells, with 0.5 cm diameter size, were made into the mass of agar medium, and then into each well were added 300 - 350 μL of enzymatic liquid culture. The plates were incubated at different temperatures.

The enzymatic liquid culture was obtained after biomass separation at 9000 rot/min, for 10 minutes, after submerged cultivation in Erlenmeyer flakes, on rotary shaker at 230 rpm and, at 20ºC temperature, for 10 days on the liquid medium with 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 pH = 7.0 [1].

Results and discussions

Starch and casein hydrolyse polar streptomycetes screening using classical techniques

Classical assay regarding screening proteases able to biosynthesis casein hydrolyses was showed through cultivation on basal Gauze agar medium with 1% casein, as unique source of nitrogen. After 12 days of cultivation at 4ºC and 25ºC, active strains identification was realized through developing a colourless zone comparing with the rest medium which is opaque. Also Casein Hydrolysis Index (SHI) was evaluated. A number of 28 *Streptomyces* sp strains, 7 strains from MIUG Collection and 21 new isolated from polar soil, East Antarctica were tested. As the results from Figure 1 show, at 25ºC new isolated strains coded S27K1G, S27K1A, 33K2G, and strains coded 1P, 11P from MIUG Collection, have high proteolitic activity. Strains coded S27K1G, 33G2G, 3KA, 3KG new isolated and also 12P, 13P from MIUG Collection show high potential to hydrolyze casein at low temperature (4ºC).

Starch hydrolyzing strains common selection was realised using radial diffusion in gel method, on Gauze medium with 1% starch, at 4°C and 25°C, during 10 days of cultivation. For starch hydrolyze zone development, the medium surface was washed with 0.1N Lugol solution, around the active colonies was created a colourless halo in comparison with the remnant medium which is blue. The results are shown in Figure 2. Strains 8K1, 3KA, S27K1G (new isolated from polar soil samples) and MIUG 1P, MIUG 11P, MIUG 12P (isolated also from polar soils but preserved in MIUG collection) have the highest ability to hydrolyze starch.

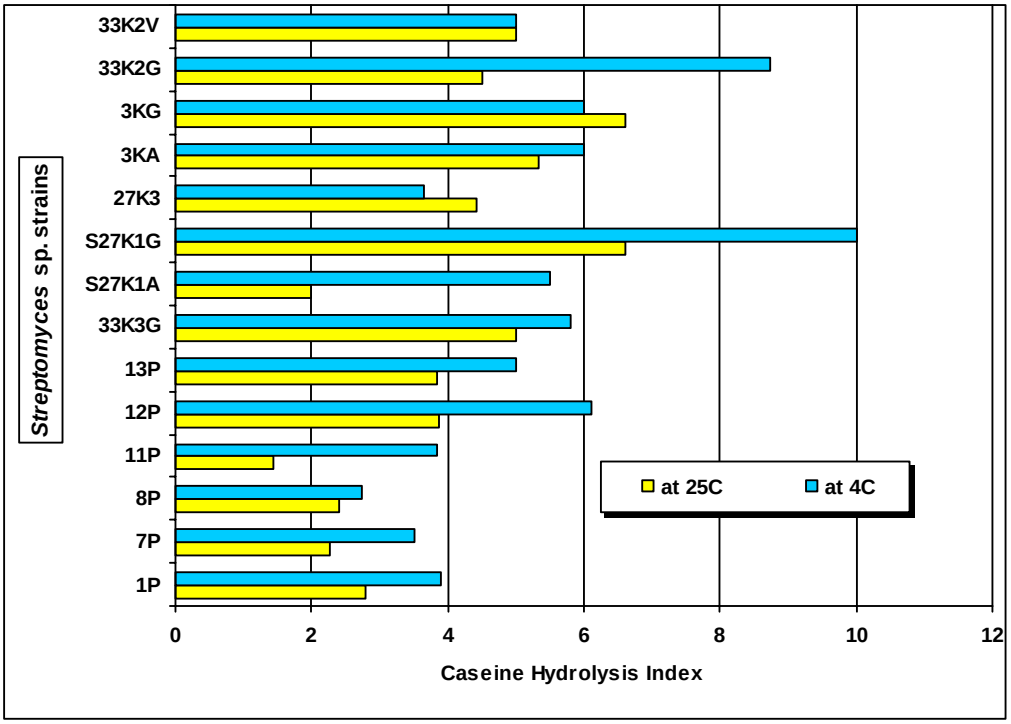

Figure 1. Casein hydrolyse potential by polar streptomycetes at low temperatures

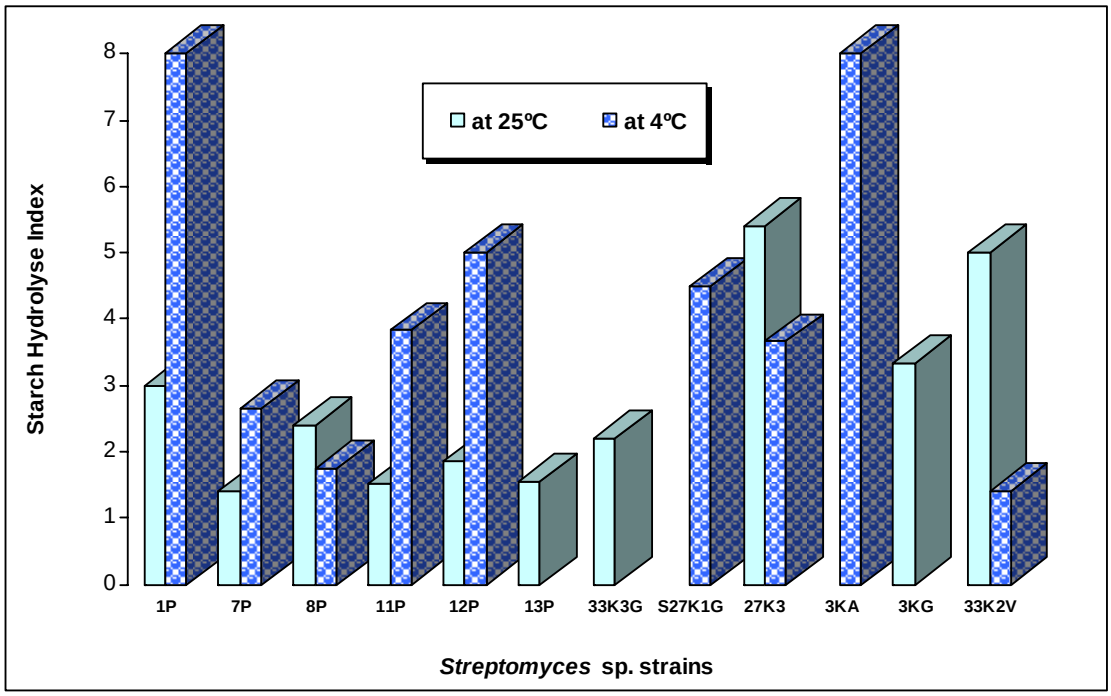

Figure 2. Starch hydrolyse potential by polar streptomycetes at low temperatures

The capacity to cold adapted enzymes production of selected strains during 10 days of cultivation on different temperatures was evaluated. At 4ºC strains 33K3, 12P and S27K1 are remarkable protease producers (Figure 3). Data shows that at 25ºC selected strains coded MIUG 7P, MIUG 12P, MIUG 13P and S27K1 are able to hydrolyze casein, after 5 days of cultivation (Figure 3).

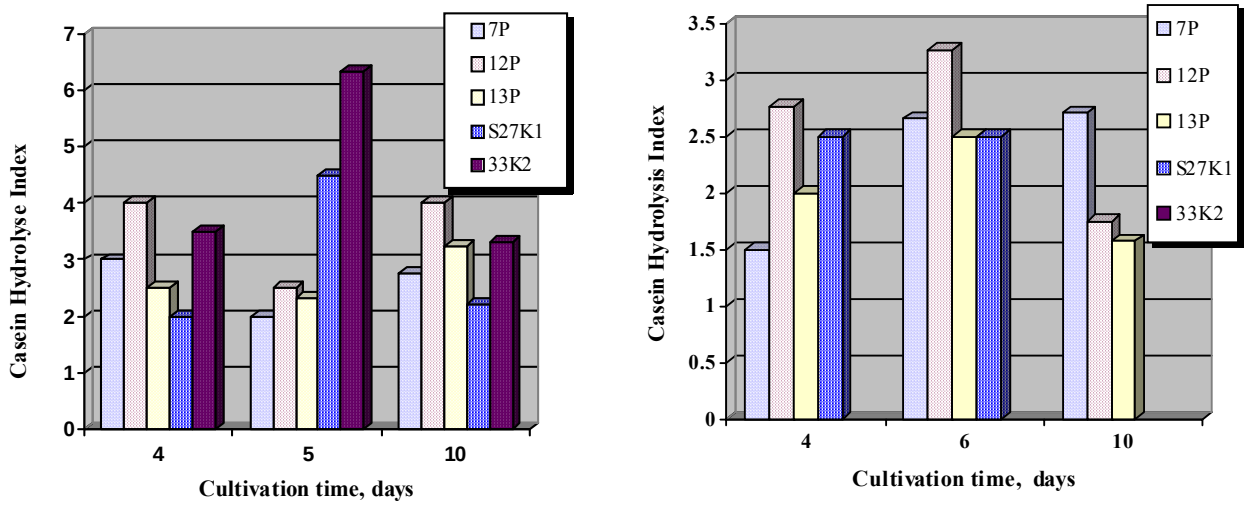

Figure 3. Dynamics of proteases biosynthesis by selected polar streptomycetes at 4 ºC (left) and 25 ºC (right)

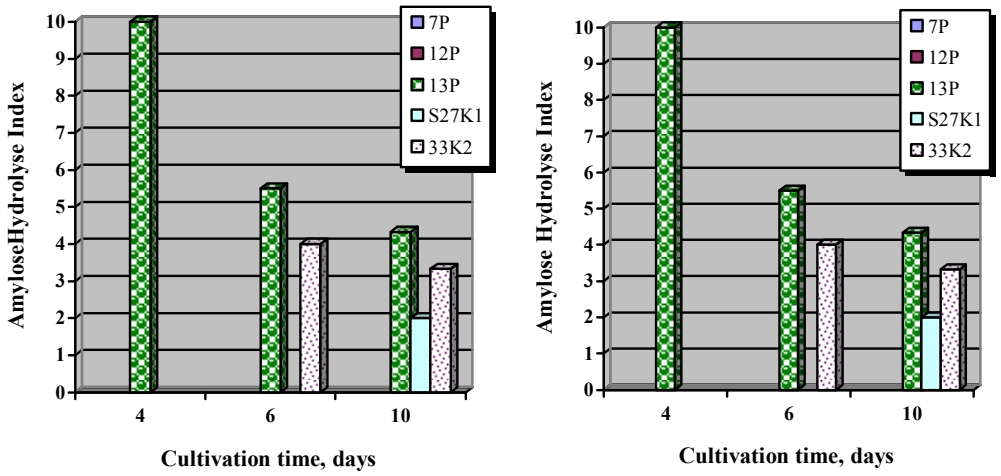

Figure 4. Dynamics of amylases biosynthesis by selected polar streptomycetes at 4ºC (left) and 25ºC (right)

Strain *Streptomyces* MIUG 13P have a high amilolitical potential at low temperature (4ºC), after only 4 days of cultivation. Data show that at 25ºC the *Streptomyces* strains MIUG 12P, 13P and 7P confirm their superiority regarding amylase potential (Figure 4). After that has been decided to test only 6 strains coded MIUG 1P, 11P, 12P, 13P due to their high hydrolase's potential and strains coded 4 Alga and P2C4.

Specialized screening by using insoluble chromogenic substrates based on AZCL (Azurine-Crosslinked)

Cold adapted amylase and protease biosynthesis potential were evaluated regarding polar streptomycetes strains metabolizing insoluble chromogenic substrates based on azurine-crosslinked with amylose or casein (AZCL-Amylose, AZCL-Casein), detectible by the blue circles around the colonies. If the bacteria produce amylases or proteases, the enzymes hydrolyse the large substrate insoluble molecules, which have been dyed with AZCL. The small hydrolyzed compounds are still dyed blue and diffuse in the plate developing blue circles zones around the colonies (Figure 5).

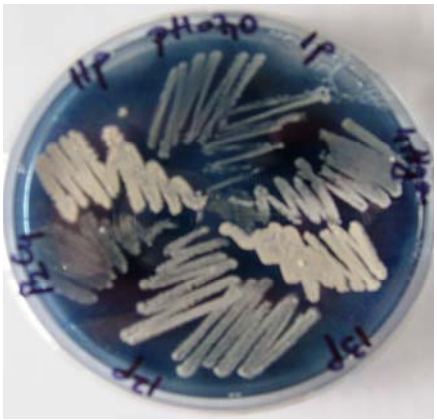

Figure 5. Growth of amylose-degrading streptomycetes on nutrient agar plate, containing 0.05% AZCL-Amylose (incubation at 20ºC and pH 7.0, for 10 days)

The chromogenic substrate AZCL-Amylose particles were degraded by all streptomycetes tested strains (4 strains from MIUG Collection coded 1P, 11P, 12P, 13P and 2 micro organisms new isolated from polar soil coded P2C4 and 4Alg). It can be seen the blue halo surrounding the colonies. This phenomenon indicates that *Streptomyces* sp. has produced hydrolases on pH 7.0.

To detect the enzymatic activity at lower temperatures and alkaline pH values the basal media supplemented with 0.05% AZCL-Amylose or 0.05% AZCL-Caseine were adjusted at the different pH values varying from pH 6.0 to pH 9.0. After the medium solidification some wells, with 0.5 cm diameter size, were made into the mass of agar medium, and then into each well were added 300 - 350 µL of enzymatic liquid culture after submerged cultivation using liquid medium, on rotary shaker at 230 rpm and, at low temperatures (4ºC and 25ºC), for 10 days. In Figure 6 and Figure 7 it can be seen that the basal medium supplemented with insoluble chromogenic substrates has the initial configuration, and then the blue zones are developing around active colony strains.

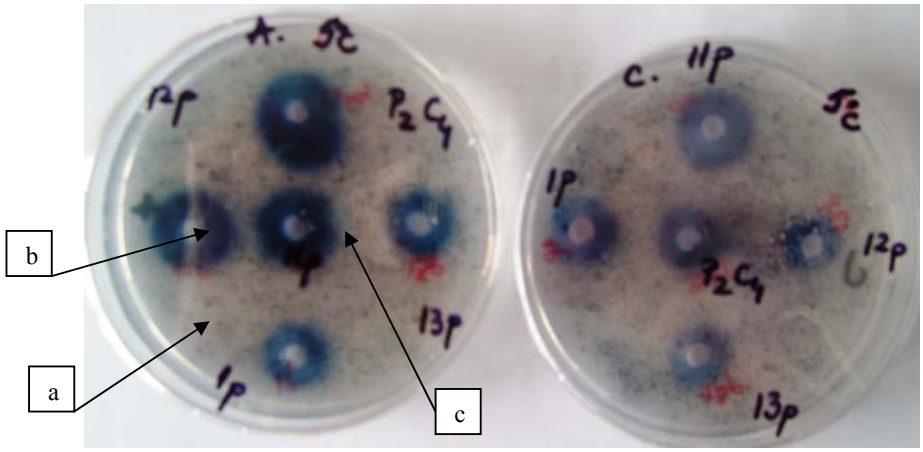

Figure 6. Amylose and casein degrading streptomycetes, at 5ºC and pH 7.0, in nutrient agar plate, containing 0.05% AZCL-Amylose or 0.05% AZCL- Casein; (a) agar medium with insoluble chromogenic substrate (blue particles wide spread into the media); (b) well with enzyme extract; (c) blue circles around the well, this means that streptomycetes are able to biosynthesis amylase (left) and protease (right)

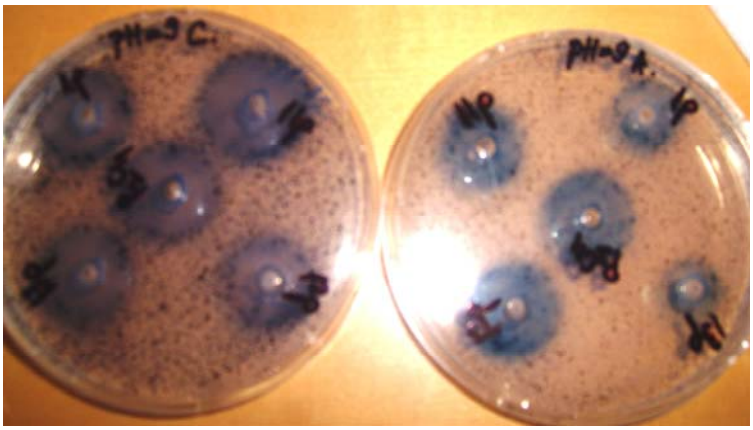

Figure 7. Detection of amylose and casein degrading streptomycetes, at pH 9.0 and 20ºC, in nutrient agar plate, containing 0.05% AZCL-Amylose (right) and 0.05% AZCL- Casein (left)

This step of screening reconfirms that all strains selected previously are high producers of hydrolase's. It can be seen that strains *Streptomyces* MIUG 1P, MIUG 11P and MIUG 13P reveal a bigger hydrolysis zone on AZCL-Casein in comparison with AZCL-Amylose.

According to the next experiment the capacity of *Streptomyces* strains to grow and to biosynthesize amylases and proteases at low temperatures and pH values were established. For tests were used cells grown on basal media supplemented with 0.05% AZCL-Amylose or 0.05% AZCL- Casein. In other experiments the enzymatic liquid culture was also tested.

According to Tables 1, 2, 3 and 4 the selected strains have the capacity to grow and to biosynthesize amylases and proteases at alkaline pH values, in both cases, when pure cultures were used (after 10-14 days of cultivation) and when the enzymatic liquid culture obtained in submerged cultivation conditions was used, too.

Strains *Streptomyces* MIUG 1P and MIUG 11P, but also new isolated P2C4 and 4Alg had a great potential to grow and to biosynthesize amylose-degradating enzymes at 15ºC and 20ºC and pH values between 6.0 and 9.0 (Tables 1 and Table 2).

Table1. Growth ability of polar selected streptomycetes on basal medium supplemented with 0.05% AZCL-Amylose agar medium at low temperatures and alkaline pH

Selected strains	Relative growth at:[a]							
	pH values				Temperatures			
	6.0	7.0	8.0	9.0	5ºC	10ºC	15ºC	20ºC
MIUG 1P	+	+	+	+	-	-	-	+
MIUG 11P	+	+	+	+	-	-	-	-
MIUG 12P	+	+	+	+	-	-	+	-
MIUG 13P	+	+	+	+	-	-	-	+
4Alg	+	+	+	+	-	-	+	+
P2C4	+	+	+	+	-	-	+	+

[a] Relative growth was scored as relative colony size on agar plates: – indicates no growth; + moderate growth and ++ good growth

Table 2. Amylase activity of the enzymatic liquid culture on AZCL-Amylose at low temperatures and alkaline pH

Selected strains	Relative amylase activity at:[b]							
	pH values				Temperatures			
	6.0	7.0	8.0	9.0	5ºC	10ºC	15ºC	20ºC
MIUG 1P	++	+	+	-	-	+	+	+
MIUG 11P	-	+	+	-	-	+	+	+
MIUG 12P	++	+	+	+	-	+	+	+
MIUG 13P	++	+	+	-	-	-	+	+
4Alg	nd	nd	nd	nd	-	+	-	-
P2C4	++	+	+	-	-	+	-	+

[b] Relative amylase activity was scored as relative SHI: – indicates no enzymatic activity; + moderate enzymatic activity and ++ good enzymatic activity; nd-untested strain

Amylases produced by tested streptomyces are active at pH values from pH 6.0 to pH 8.0 and low temperatures from 10ºC to 20ºC.

Most of studied polar streptomycetes are able to grow and to biosynthesize proteases at alkaline pH but not at low temperatures. In contrast the enzymes are active at alkaline pH and low temperatures (Tables 3 and Table 4).

Table 3. Growth ability of polar selected streptomycetes on basal medium supplemented with 0.05% AZCL-Casein agar medium at low temperatures and alkaline pH

Selected strain	Relative growth at:[c]							
	pH values				Temperatures			
	6.0	7.0	8.0	9.0	5ºC	10ºC	15ºC	20ºC
MIUG 1P	-	-	+	+	-	-	-	-
MIUG 11P	-	+	+	+	-	-	-	-
MIUG 12P	-	+	+	-	-	-	-	+
MIUG 13P	-	-	-	-	-	-	-	-
4Alg	+	+	+	+	-	-	+	+
P2C4	+	+	+	+	-	-	-	+

[c] Relative growth was scored as relative colony size on agar plates: – indicates no growth; + moderate growth and ++ good growth

Table 4. Protease activity of the enzymatic liquid culture on AZCL-Casein at lower temperatures and alkaline pH

Selected strain	Relative protease activity[d]							
	pH values				Temperatures			
	6.0	7.0	8.0	9.0	5ºC	10ºC	15ºC	20ºC
MIUG 1P	-	+	++	++	-	+	+	+
MIUG 11P	++	+	++	++	-	+	+	+
MIUG 12P	++	+	++	+	-	-	+	+
MIUG 13P	-	-	-	-	-	-	-	-
4Alga	nd	nd	nd	nd	-	-	-	-
P2C4	++	++	++	++	-	+	+	+

[d] Relative protease activity was scored as relative SHI: – indicates no enzymatic activity; + moderate enzymatic activity and ++ good enzymatic activity; nd- untested strain.

The rate of substrates hydrolysis was also tested. By using 300 - 350 µL of enzymatic liquid culture, poured in the wells; after 24 h the substrate hydrolysis potential was evaluated. The plates were incubated at different temperatures. The strains coded MIUG 1P, MIUG 11P, MIUG 12P and P2C4 show a high hydrolytically-degrading potential after 24 h at lower temperatures (5-20ºC). At alkaline pH *Streptomyces* coded MIUG 1P, MIUG 11P, MIUG 12P, MIUG 13P and P2C4 were able to synthesize a good level of amylases and proteases (Table 5 and Table 6).

Table 5. Amylase activity release of enzymatic liquid culture of polar streptomycetes on AZCL-Amylose after 24 h of preservation at low temperatures and different pH values

	Relative amylase activity:[e]							
Selected strain	**pH values**				**Temperatures**			
	6.0	**7.0**	**8.0**	**9.0**	**5ºC**	**10ºC**	**15ºC**	**20ºC**
MIUG 1P	+	++	+	++	+	+	+	++
MIUG 11P	++	++	+	++	++	+	+	++
MIUG 2P	++	++	++	++	++	++	++	++
MIUG 13P	+	++	+	+	+	+	+	+
4Alg	-	-	-	-	-	-	-	-
P2C4	++	++	++	++	++	++	++	++

[e] Relative amylase activity was scored as relative SHI; - indicates no enzymatic activity; + moderate enzymatic activity and ++ good enzymatic activity

Table 6. Protease activity release of enzymatic liquid culture of polar streptomycetes on AZCL-Casein after 24 h of preservation at low temperatures and different pH values

	Relative protease activity:[f]							
Selected	**pH values**				**Temperatures**			
strain	**6.0**	**7.0**	**8.0**	**9.0**	**5ºC**	**10ºC**	**15ºC**	**20ºC**
MIUG 1P	++	++	++	++	+	++	++	++
MIUG 11P	++	++	++	++	++	++	++	++
MIUG 2P	+	+	+	++	+	+	+	+
MIUG 13P	+	+	+	++	+	+	+	+
4Alg	-	-	-	-	-	-	-	-
P2C4	++	++	++	++	++	+	++	+

[f] Relative protease activity was scored as relative SHI; - indicates no enzymatic activity; + moderate enzymatic activity and ++ good enzymatic activity

The results certify the streptomycetes ability to produce amylases and proteases cold adapted and alkaline pH actives. If the hydrolysis time had been longer, a SHI = 4-5 could have been obtained after 6 days for polar strains from MIUG Collection.

Studied polar streptomycetes having polymers-degrading activities can be successfully used in bioremediation process, dairy industry, bakery and detergents making, at low temperatures.

Conclusions

Developed plate technique provides unique possibility for active enzymes producing micro organism's selection straight in the primary screening.

Using for screening agar plates supplemented with insoluble AZCL can be screen streptomycetes able to produce specific enzymes, alpha-amylase and endo-protease as a more specific technique.

The major advantages of the screening agar plate technique can be summarized as follows: different polymers-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 polymers-degrading activities

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