PARTIAL CHARACTERIZATION OF COLD ACTIVE AMYLASES AND PROTEASES OF \textit{STREPTOMYCES} SP. FROM ANTARCTICA

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\textbf{ABSTRACT}

The aim of this study was to isolate novel enzyme-producing bacteria from vegetation samples from East Antarctica and also to characterize them genetically and biochemically in order to establish their phylogeny. The ability to grow at low temperature and to produce amylases and proteases cold-active was also tested. The results of the 16S rRNA gene sequence analysis showed that the 4 Alga rRNA was 100% identical to the sequences of \textit{Streptomyces} sp. rRNA from Norway and from the Solomon Islands. The \textit{Streptomyces} grew well in submerged system at 20°C, cells multiplication up to stationary phase being drastically increased after 120 h of submerged cultivation. The beta-amylase production reached a maximum peak after seven days, while alpha-amylase and proteases were performing biosynthesis after nine days of submerged cultivation at 20°C. Newly \textit{Streptomyces} were able to produce amylase and proteases in a cold environment. The ability to adapt to low temperature of these enzymes could make them valuable ingredients for detergents, the food industry and bioremediation processes which require low temperatures.

\textbf{Key words:} \textit{Streptomyces} sp., East Antarctica, cold-adapted strain, cold active proteases and amylase

\textbf{INTRODUCTION}

Extremozymes have great economic potential in many industrial processes - detergents, textiles, baking, brewing, starch, animal food, leather and the pulp industries, including agricultural, chemical, and pharmaceutical applications (15, 5). Among extremozymes, cold-active enzymes have important biotechnological applications in the food industry, as well as in biomass conversion and bioremediation. Running processes at low temperatures reduces risk of contamination by mesophiles and also saves energy (33, 16, 13, 28, 27).

Therefore, researchers are now trying to exploit extremophiles as valuable source of novel enzymes (6). Particular enzymes who display highly specific activity and catalytic efficiency at low temperatures have the capability to adapt to cold habitats. (31). Among the extremophiles, cold-
adapted organisms such as 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°C, whereas psychrophilic organisms grow faster at a temperature of 15°C or lower, but are unable to grow above 20°C (26, 25, 31).

Amylases (endo-1,4-α-D-glucanohydrolases EC 3.2.1.1) represent 25% of the sales in enzyme market. These enzymes play a vital role in many industrial manufacturing processes such as textile, paper, food industries and detergents (6, 18, 35, 38). Proteases are industrially important enzymes also having wide applications in the manufacture of pharmaceuticals, leather, silk, detergents, food, the waste processing industries, diagnostics and for the recovery of silver used in X-ray films (33, 20).

The possibility of using actinomycetes, especially *Streptomyces* for enzyme production has recently been investigated. *Streptomyces* genus exhibits remarkable capacity for the synthesis of many valuable enzymes with attractive properties (39, 6, 38). *Streptomyces* species have served as an important source of numerous secondary metabolites, enzymes (9) and antibiotics (1) mainly due to their shorter generation time, and the ease of genetic and environmental manipulation. Literature offers valuable information on streptomycetes species able to produce proteases. In particular *S. clavuligerus, S. griseus, S. moderatus, S. rimosus, S. thermoviolaceus, S. thermovagaris, S. avermectins*, and *S. pactum DSM 40530* (36, 1, 23), *S. gulbargensis* (10) are able to produce proteases, while *S. fradie, S. griseus, S. lividans, S. peptidofaciens*, and *S. rinosus* are known as aminopeptidase producers. Alpha-amylases have been also produced with *S. aureofaciens, S. rinosus, S. gulbargensis, S. hygroscopicus, S. preacox* (12, 34, 40). The study of transglutaminase production by *Streptomyces higroscopus, Streptomyces mobaraensis, S. libani, Streptoverticillium mobaraense* is more recent (11, 37, 24, 30, 8).

Due to the insufficiency of information regarding useful cold-active enzymes and the industrial application of the psychrophilic and psychrotrophic taxa it is imperative to isolate new cold-adapted bacteria and to identify production of cold-adapted amylase and proteases.

Herein we characterize genetically and biochemically a polar streptomycete 4 Alga, we examine its growth behaviour and also the production of amylases and proteases at low temperature.

**MATERIALS AND METHODS**

**Microorganism and growth properties**

The selected bacterial coded 4 Alga was isolated from Antarctic vegetation samples from Progress Lake 2, Larsemann Hills, East Antarctica (Negoita T. Gh.) (7). The stock cultures were maintained on Gause-agar medium with the following composition (g/l): starch 20, K₂HPO₄ 0.5, MgSO₄·7H₂O 0.5, KNO₃ 1.0, NaCl 0.5, FeSO₄·7H₂O 0.01, agar 25.0, pH 7.2–7.4 (6).

Growth kinetic and enzyme biosynthesis were performed in a submerged system. A liquid medium with following composition (g/l): soluble starch 20.0, corn steep liquor 10.0, (NH₄)₂SO₄ 6.0, CaCO₃ 8.0, NaCl 5.0 and soybean oil 0.2 ml, pH 7.0 (7) was inoculated with 2% spore suspension and incubated during 15 days, at 20°C and 7.276x g. Growth kinetic was acquired by measuring the cell density at a wavelength of 610 nm with spectrophotometer UV-VIS, JENWAY.

**Morphological and biochemical characterization**

The morphology of the 4 Alga strain was assessed by the colonies formed on Gause agar medium and by contrast phase microscopy using Olympus equipment (Japan). The
biochemical properties were characterised by using the Biolog microtiter plate in accordance with the manufacturer’s instructions. The results were interpreted as positive reaction (+) or negative reaction (-) after 24 h incubation at 20°C.

**16S rRNA gene amplifications and sequencing**

Genomic DNA was isolated from the 4 Alga strain using the Power Soil® DNA Isolation Kit (Mo Bio Laboratories, Inc.). Isolation of the 16S rRNA gene was carried out by means of PCR with the primer pair 27F/1492R (22). PCR was performed in a 25 µl reaction mixture containing 2.5 µl 10X buffer (without MgCl₂), 1.25 µl MgCl₂ (25 mM), 0.25 µl dNTP (25 mM), 0.25 µl Taq polymerase (5 units/µl) (Ampliqon), 1.0 µl of each of the primers 27 F and 1492R. Amplification was performed with an initial denaturising step (5 min at 94ºC), followed by 30 cycles of 94ºC for 30 sec., 55ºC for 1 min and 72ºC for 1 min and a final extension at 72ºC for 5 min. The amplified fragments were purified from agarose gels using QIAquick gel extraction kit (QIAGEN) and inserted into the cloning vector pCR®2.1 TOPO according to the manufacturer’s instructions. Inserts in pCR®2.1 TOPO were sequenced at GATC Biotech AG, Germany, and the DNA sequences were submitted to GenBank/EMBL/DDBJ with the accession numbers EF571003, EF571002, EU263063 and GQ924533.

**Phylogenetic analysis**

Related sequences were retrieved from public databases using BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/blast/). The closest related 16S rRNA gene sequences were aligned with the sequences of the Antarctic isolate using the alignment algorithm in the CLC Main Workbench 5.1 (CLC bio, Denmark).

**Amylase and proteases assays**

The crude extracts were obtained after cultivation in submerged conditions and biomass separation at 4952x g, for 15 minutes. Proteolytic assay was performed via modified Anson method using 2% casein as substrate (2) and proteases 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 20ºC) hydrolyzed the casein to release, in one minute, the hydrolysis products soluble in the trichloroacetic acid; this provides coloration equivalent, measured as absorbance at 670 nm, to 1 µmol of tyrosine, in the presence of the Folin-Ciocalteau reagent by using a tyrosine standard curve over the range of 0.02-0.24 µmol/ml (14).

Alpha-amylase assay was performed by using an adapted method based on a selective distinction of the hydrolysis products in 0.1 N Lugol solution. One α-amylase unit represents the equivalent of the amount of enzyme which generates a 0.05 decrease in the optical density, for 1 min, measured at 610 nm, of the colour iodine-starch complex, into a 1% starch solution, at pH 7.0 and 20ºC (4).

The Shaffer-Somogyi method was used to measure the released maltose with few modifications (32). One β-amylase unit represents the amount of maltose (in mg) produced by one ml crude extract by using 1% starch as substrate, at 20ºC and pH 7.0 for 1 min.

**RESULTS**

**Phylogenetic analysis**

By cultivation on Gause agar medium the outer surface of colonies was perfectly round initially, but after the fourth day of incubation started to develop white aerial mycelium that appeared powdery and also formed spores. Distinctive substrate mycelium colours were not produced and diffusible extracellular pigment was not formed. Microscopic examination revealed that aerial mycelia were morphologically branched with compact spirals of sporophore.

After genomic DNA separation and purification, the sequence of the 16S rRNA gene was determined in order to achieve the phylogeny. Search for similar sequences in the
GenBank/EMBL/DDBJ database and subsequent alignment of the retrieved sequences indicated that the DNA sequence showed high similarity (99-100%) to the known 16S rRNA gene sequences from *Streptomyces*. The 16S rRNA gene sequence of 4 Alga strain was shown to be 100% identical to sequences of *Streptomyces* sp. isolates from Norway (accession no. EU263063) and from Solomon Islands (accession no. GQ924533) and to other *Streptomyces* (code P2C4 and MIUG 12P members of The Collection of Industrial Microbiology Laboratory of University of Galați Romania) (Fig. 1 and Table 1).

**Figure 1.** Phylogenetic tree of the 16S rDNA sequence of 4 Alga. The tree was rooted via a neighbor-joining method, and numbers within the dendogram indicate the occurrence (%) of the branching order in 100 bootstrapped trees.

| Table 1. Similarity of 16S rRNA gene sequences in the GenBank/EMBL/DDBJ database |
|---------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
|                                 | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
| Streptomyces 4 Alga             |     |     |     |     |     |     |     |     |     |     |
| EF571003                        | 99.93 | 99.93 | 99.93 | 99.93 | 100.0 | 100.0 | 99.80 | 99.80 | 99.60 |
| EF571002                        | 99.93 | 100.0 | 100.0 | 100.0 | 99.93 | 99.93 | 99.93 | 99.93 | 99.93 |
| AF429394                        | 99.93 | 100.0 | 100.0 | 100.0 | 99.93 | 99.93 | 99.93 | 99.93 | 99.93 |
| EU443837                        | 99.93 | 100.0 | 100.0 | 100.0 | 99.93 | 99.93 | 99.93 | 99.93 | 99.93 |
| EU263063                        | 100.00 | 99.93 | 99.93 | 99.93 | 99.93 | 100.0 | 99.80 | 99.80 | 99.60 |
| GQ92453                         | 100.00 | 99.93 | 99.93 | 99.93 | 99.93 | 100.0 | 99.80 | 99.80 | 99.60 |
| Streptomyces P2C4               | 99.80 | 99.73 | 99.73 | 99.73 | 99.73 | 99.73 | 99.73 | 99.73 | 99.73 |
| Streptomyces MIUG 12P           | 99.60 | 99.53 | 99.53 | 99.53 | 99.53 | 99.53 | 99.53 | 99.53 | 99.53 |

Biochemical characterization of the *Streptomyces* sp. 4 Alga isolate

The biochemical properties of *Streptomyces* 4 Alga were assessed based on their ability to oxidize various carbon sources of the Biolog microtiter plate assay (Table 2). Arabinose, xylose, mannitol, L-phenylalanine were metabolised by the 4 Alga strain. There are many reports regarding utilization of carbon sources by the streptomycetes and in this sense Vonothini et al., (2008) research concerning actinomycete strain, PS-18A isolated from an estuarine shrimp pond sustain our results. Fructose, maltose, dextrin, glycogen, Tween 40, Tween 80, L-arabinose, L-aspartic acid, L-leucine, glyceral,
citric acid, itaconic acid, D-mannitol, L-histidine, D-serine, L-threonine were well utilized; sucrose, inositol, trehalose, formic acid, malonic acid, L-alanine, inosine, hydroxy-L-proline were not utilized. These results are also in accordance with Kim et al., (1998) for *Streptomyces thermocarboxydivorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., two moderately thermophilic carboxydotrophic species from soils.

| Principal component | Reactions | Principal component | Reactions |
|---------------------|-----------|---------------------|-----------|
| α-cyclodextrin      | +         | L-Rhamnose         | -         |
| Dextrin             | +         | D-Sorbitol         | -         |
| Glycogen            | +         | Sucrose            | -         |
| Tween 40            | +         | D-trehalose        | -         |
| Tween 80            | +         | Turanose           | -         |
| N-acetyl-D-galactosamine | +     | Xylitol            | +         |
| N-acetyl-D-glucosamine | +     | Pyruvic acid methyl ester | + |
| Adonitol            | +         | Succinic Acid Mono-Methyl-Ester | + |
| L-Arabinose         | +         | Acetic Acid        | +         |
| D-Arabitol          | +         | cis-Aconitic Acid  | +         |
| D-Cellobiose        | +         | Citric Acid        | +         |
| i-Erythritol        | +         | Formic Acid        | -         |
| D-Fructose          | +         | D-Galactonic Acid Lactone | - |
| L-Fucose            | +         | D-Galacturonic Acid | -         |
| D-Galactose         | +         | D-Glucuronic Acid  | -         |
| Gentiobiose         | -         | D-Glucosaminic Acid | -         |
| α-D-Glucose         | -         | D-Glucuronic Acid  | +         |
| m-Inositol          | -         | α-hydroxybutyric Acid | + |
| α-D-Lactose         | -         | β-hydroxybutyric Acid | + |
| Lactulose           | +         | γ-hydroxybutyric Acid | + |
| Malose              | +         | p-hydroxy Phenylacetoid Acid | + |
| D-Mannitol          | +         | Itaconic Acid      | +         |
| D-Mannose           | +         | α-Keto Butyric Acid | +         |
| D-Melibiose         | +         | Hydroxy-L-Proline  | -         |
| β-Methyl D-glucoside| +         | L-Leucine          | +         |
| D-Psicose           | +         | L-Orithine         | +         |
| D-Raffinose         | -         | L-Phenylalanine    | +         |
| α-Keto Glutaric Acid| -         | L-Proline          | +         |
| α-Keto Valeric Acid | -         | L-Pyroglutamic Acid | +         |
| D,L-Lactic Acid     | -         | D-serine           | +         |
| Malonic Acid        | -         | L-Serine           | +         |
| Propionic Acid      | -         | L-Threonine        | +         |
| Quinic Acid         | +         | D,L-Carnitine      | +         |
| D-Saccharic Acid    | +         | γ-Amino Butyric Acid | + |
| Sebacic Acid        | +         | Urocanic Acid      | +         |
| Succinic Acid       | +         | Inosine            | -         |
| Bromosuccinic Acid  | +         | Uridine            | +         |
| Succinamic Acid     | +         | Thymidine          | +         |
| Glucuronamide       | +         | Phenylethanol-amine | +     |
| L-Alaminamide       | -         | Putrescine         | +         |
| D-Alanine           | -         | 2-Aminooethanol    | +         |
| L-Alanine           | -         | 2,3-Butanediol     | +         |
| L-Alanyl-glycine    | -         | Glycerol           | +         |
| L-Asparagine        | -         | D,L-α-Glycerol Phosphate | + |
| L-Aspartic Acid     | +         | α-D-Glucose 1-Phosphate | + |
| L-Glutamic Acid     | +         | D-Glucose 6-Phosphate | + |
| Glycyl-L-Aspartic Acid | +    | L-Histidine        | +         |
| Glycyl-L-Glutamic Acid | +    |                     |           |

+ Positive reaction; - negative reaction
Growth kinetic and time course production of amylases and protease in cold conditions

The *Streptomyces* 4 Alga is a psychrotolerant strain (data not shown). The data presented in Fig. 2 showed that the *Streptomyces* 4 Alga was capable to adapt easily to submerged cultivation and that cells multiplication drastically increased in the exponentially growth phase and reached the stationary phase after the five days of submerged cultivation at 20°C. Previous research (Thumar and Singh, 2007) showed that *Streptomyces clavuligerus* strain mit-1 reached the stationary phase after 100 h. It can be seen that the optical density is high compared to literature results for *Streptomyces* sp. D1 from marine sediment samples, which reached the stationary phase after almost six days of submerged cultivation at 45°C and 5.502 x g (Chakraborty et al., 2008).

![Graph showing growth kinetic of Streptomyces 4 Alga](image)

**Figure 2.** Growth kinetic of *Streptomyces* 4 Alga in submerged culture, by shaking at 7.276 x g. Initial pH 7.0; inoculum 2% spore suspension; incubation period: 15 days; incubation temperature 20°C. Data are the average of two parallel replicates.

The production of amylases and proteases was achieved during 15 days of submerged culture at 20°C. Alpha-amylase was synthesised during the early exponential phase, however the highest yield (14.10 UA) was registered after 216 h of cultivation, in the middle of the stationary phase. After that the amylase biosynthesis potential remains constant and after 12 days of cultivation amylase biosynthesis slowly decreases (Fig. 3).

These results are in accordance with Chakraborty et al. (2008) who reported that amylase from *Streptomyces* sp. D1, from marine sediments sampled from coastal region India, reached the optimum production after 11 days of submerged cultivation at 45°C and 5.502 x g. Syed et al., (2009), referred that amylase from *S. gulbargensis* DAS 131 reached the maximum of enzyme activity after 48 h of incubation at 28°C.

Beta-amylase synthesis started in the middle of the stationary phase. The highest production of beta-amylase, 173.47 UA, was achieved after 168 h of submerged cultivation, while alpha-amylase biosynthesis was noticed after 216 h of submerged cultivation (Fig. 4).

The maximum proteases biosynthesis was recorded after nine days of cultivation during the stationary phase (Fig. 5). This result is in agreement with Bascaran et al. (1990) and Moreira et al. (2001) who showed that biosynthesis of
proteases from *Streptomyces clavuligerus* starts in the early stationary phase of growth. Filamentous bacteria like *Streptomyces rimosus* and *Streptomyces clavuligerus* mit-1 recorded the optimum proteases production at 166 h (40) and 144 h (36) respectively. These results certify the ability of newly *Streptomyces* 4 Alga to grow well at low temperature (20°C) and to produce cold-active amylase (alpha and beta amylase) and proteases.

![Alpha-amylase production](image1)

**Figure 3.** Alpha-amylase production at *Streptomyces* 4 Alga in submerged culture, by shaking at 7.276x g. Initial pH 7.0; inoculum 2% spore suspension; incubation period: 15 days; incubation temperature 20°C. Data are the average of two parallel replicates.

![Beta-amylase production](image2)

**Figure 4.** Beta-amylase production at *Streptomyces* 4 Alga in submerged culture, by shaking at 7.276x g. Initial pH 7.0; inoculum 2% spore suspension; incubation period: 15 days; incubation temperature 20°C. Data are the average of two parallel replicates.
DISCUSSION

The main objective of this study was to isolate novel enzyme-producing filamentous bacteria from vegetation samples from East Antarctica, to characterize them genetically and biochemically, in order to establish their phylogeny and their ability to grow at low temperature, and to generate cold-adapted amylase and proteases.

The 16S rRNA gene sequence of the new strain 4 Alga was shown to be 100% identical to the sequences of *Streptomyces* sp. from Norway and from the Solomon Islands. The morphological and biochemical studies also confirmed the classification of this strain in *Streptomyces* genus. Therefore, in accordance with the GenBank, the strains closely related with the 4 Alga are psychrophiles isolated from cold Arctic and Antarctic regions. *Streptomyces* sp. MP47-06 (EU263062.1) showed a 99% similarity with the 4 Alga strain and it was isolated from a Norway fjord (19).

While most of the studies are concerned about the molecular phylogeny of streptomycetes, similar attention has not been paid to their enzymology. Their ability to produce a variety of enzymes may be an attractive phenomenon in these prokaryotes (36).

A fast and accurate method has been widely used to identify newly strain using 16S r DNA gene sequence analysis. This technique is important to identify organisms which are slow-growing, fastidious and where identification by conventional methods is time consuming along with subjective interpretation such as identifying streptomycetes. Castillo et al. (2003) had *Streptomyces* sp. NRRL 30566, which produced novel antibiotic Kakadumycins using this method (17).

The *Streptomyces* 4 Alga strain grew properly at low temperatures. The data showed that the *Streptomyces* 4 Alga was capable to easily adapt to submerged cultivation and reached the stationary phase after the five days of submerged cultivation at 20°C.

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Figure 5. Proteases production at *Streptomyces* 4 Alga in submerged culture, by shaking at 7.276x g. Initial pH 7.0; inoculum 2% spore suspension; incubation period: 15 days; incubation temperature 20°C. Data are the average of two parallel replicates.
Alpha-amylase production was recorded after 216 h of cultivation, in the middle of the stationary phase. After that the amylase biosynthesis potential remains constant and after 12 days of cultivation amylase biosynthesis slowly decreased. Beta-amylase synthesis started in the middle of the stationary phase and it was achieved after 168 h of submerged cultivation. The maximum proteases biosynthesis was revealed after nine days of submerged cultivation. These results are in accordance with previously reported literature results (Yang and Wang, 2000) which also confirm that the biosynthesis of proteases was detected before amylase production, at *Streptomyces rimosus*.

*Streptomyces* 4 Alga was highly competent to grow in cold conditions and to biosynthesize amylase and proteases cold-adaptive at low temperature (20°C).

The amylases and proteases generated by this cold-adapted streptomycete strain could be valuable candidates in competitive bioremediation of polluted soils and waste waters when the metabolic ability of the endogenous microflora is reduced due to low temperatures. These enzymes might also have tremendous applications in other industrial processes, which require low temperatures.

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