PARTIAL CHARACTERIZATION OF COLD ACTIVE AMYLASES AND PROTEASES OF *STREPTOMYCES* SP. FROM ANTARCTICA

Mihaela Cotârleţ[1], Teodor Gh. Negoiţă[1], Gabriela E. Bahrim[2], Peter Stougaard[3]

[1]Romanian Polar Research Institute, Bucharest, Romania; [2]University "Dunărea de Jos", Faculty of Food Science and Engineering, Bioengineering Department, Galaţi, Romania; [3]University of Copenhagen, Faculty of Life Sciences, Department of Agriculture and Ecology, Denmark.

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 *Streptomyces* sp. rRNA from Norway and from the Solomon Islands. The *Streptomyces* grew well in submerged system at 20ºC, 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 20ºC. Newly *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.

Key words: *Streptomyces* sp., East Antarctica, cold-adapted strain, cold active proteases and amylase

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 20ºC, whereas psychrophilic organisms grow faster at a temperature of 15ºC or lower, but are unable to grow above 20ºC (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. thermovulgaris, S. avermectinus*, 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. rimosus* are known as aminopeptidase producers. Alpha-amylases have been also produced with *S. aureofaciens, S. rimosus, 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, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, KNO_3 1.0, NaCl 0.5, $FeSO_4 \cdot 7H_2O$ 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, $(NH_4)_2SO_4$ 6.0, $CaCO_3$ 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 $MgCl_2$), 1.25 µl $MgCl_2$ (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 94°C), followed by 30 cycles of 94°C for 30 sec., 55°C for 1 min and 72°C for 1 min and a final extension at 72°C 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 20°C) 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 20°C (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 20°C 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).
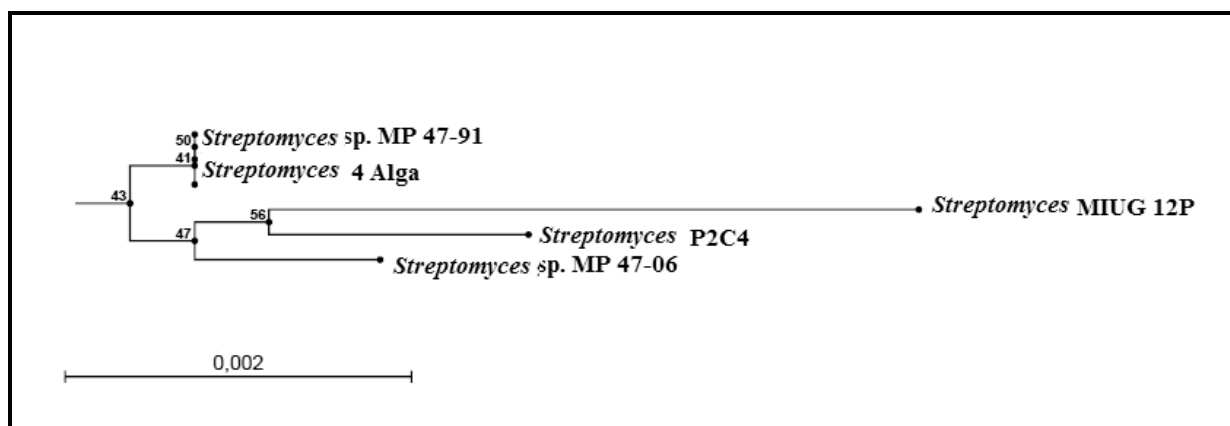

Figure 1. Phylogenetic tree of the 16S rDNA sequence of 4 Alga. The tree was rooted via a neighbor-joining method, and numbers within the dendogram indicate the occurrence (%) of the branching order in 100 bootstrapped trees.

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		99.93	99.93	99.93	99.93	100.0	100.0	99.80	99.80	99.60
EF571003	99.93		100.0	100.0	100.0	99.93	99.93	99.87	99.73	99.53
EF571002	99.93	100.0		100.0	100.0	99.93	99.93	99.87	99.73	99.53
AF429394	99.93	100.0	100.0		100.0	99.93	99.93	99.87	99.73	99.53
EU443837	99.93	100.0	100.0	100.0		99.93	99.93	99.87	99.73	99.53
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
EU263062.1	99.80	99.87	99.87	99.87	99.87	99.80	99.80		99.73	99.53
Streptomyces P2C4	99.80	99.73	99.73	99.73	99.73	99.80	99.80	99.73		9953
Streptomyces MIUG 12P	99.60	99.53	99.53	99.53	99.53	99.60	99.60	99.53	99.53	

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

The biochemical properties of *Streptomyces* 4 Alga were assesed 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, glycerol,

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 thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov., two moderately thermophilic carboxydotrophic species from soils.

Table 2. Biochemical properties of the *Streptomyces* 4 Alga after 24 h of incubation at 20°C

Principal component	Reaction	Principal component	Reaction
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-Gluconic 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	+
Maltose	+	p-hydroxy Phenylacetic 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	+	Phenyethyl-amine	+
L-Alaninamide	-	Putrescine	+
D-Alanine	-	2-Aminoethanol	+
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 5.502x g (Chakraborty *et al.*, 2008).
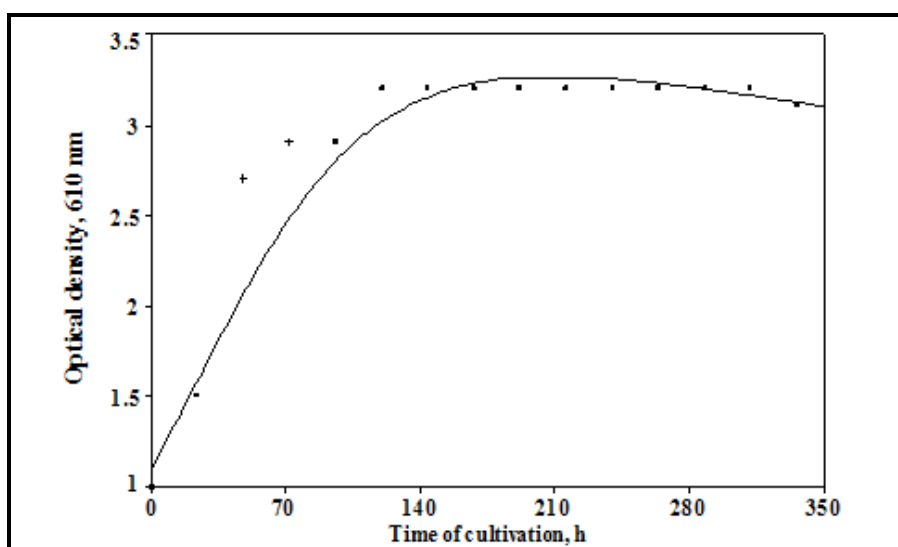
Figure 2. Growth kinetic of *Streptomyces* 4 Alga in submerged culture, by shaking at 7.276x g. Initial pH 7.0; inoculum 2% spore suspension; incubation period: 15 days; incubation temperature 20°C. Data are the average of two parallel replicates.

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 5.502x 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.
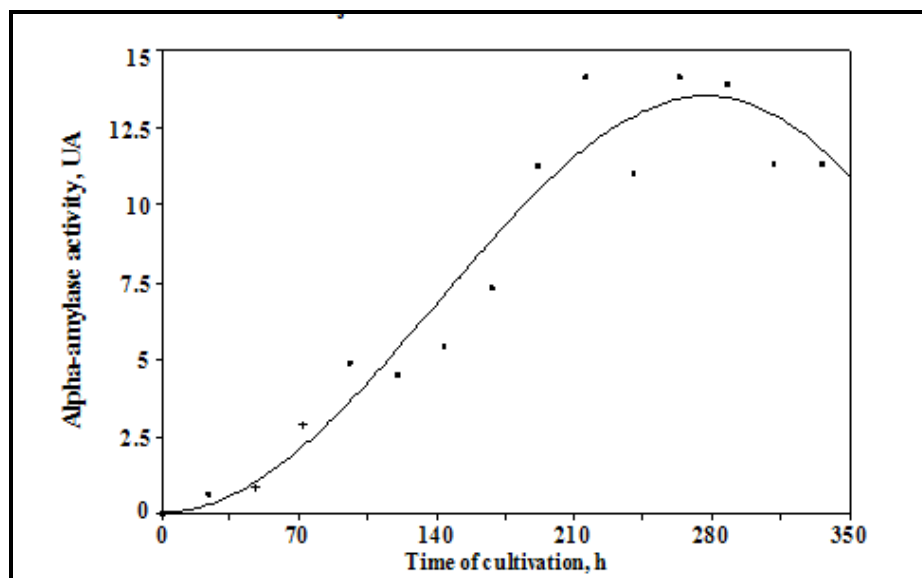

Figure 3. Alpha-amylase production at *Streptomyces* 4 Alga in submerged culture, by shaking at 7.276x g. Initial pH 7.0; inoculum 2% spore suspension; incubation period: 15 days; incubation temperature 20°C. Data are the average of two parallel replicates.
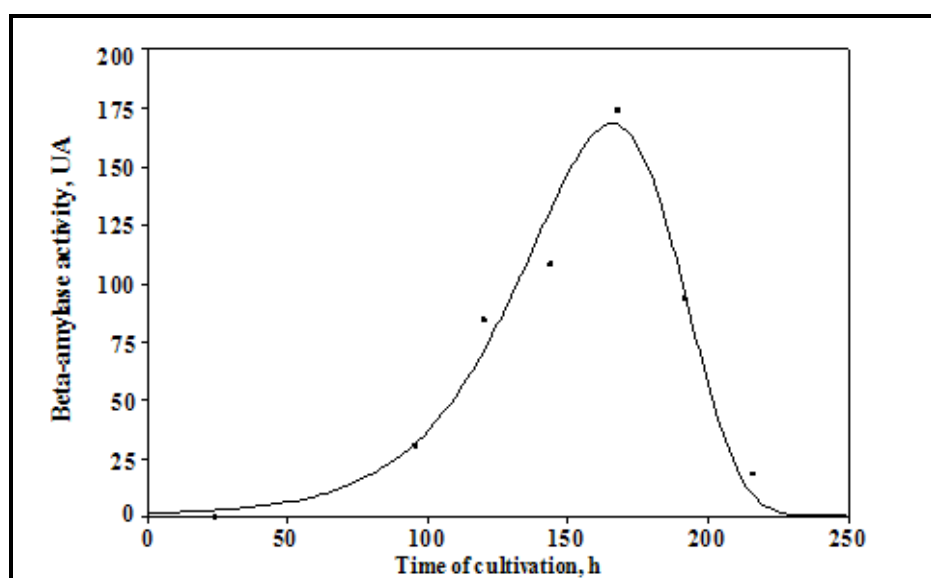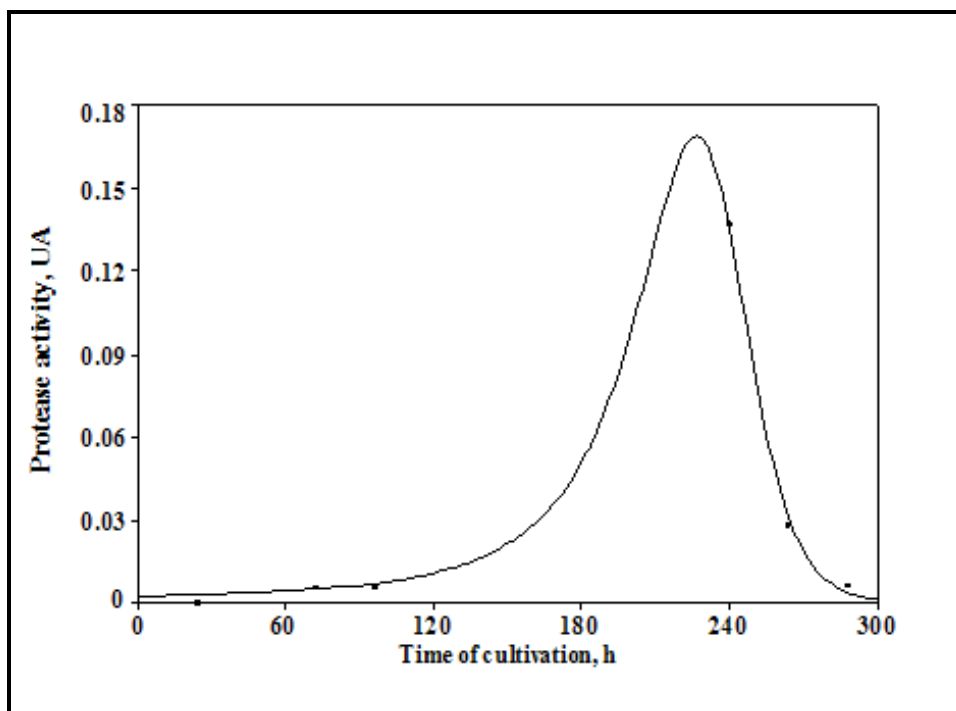

Figure 4. Beta-amylase production at *Streptomyces* 4 Alga in submerged culture, by shaking at 7.276x g. Initial pH 7.0; inoculum 2% spore suspension; incubation period: 15 days; incubation temperature 20°C. Data are the average of two parallel replicates.

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.

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.

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 (20ºC).

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.

REFERENCES

1. Ahmed, S.A.; Ramadan, A.A.; El-Shayeb, N.M.A.; Radwan H.H.; Saleh, S.A. (2008). Optimization, Immobilization of Extracellular Alkaline Protease and Characterization of its Enzymatic Properties. Res. *J. Agricul. Biol. Sci*. 4, 434-446.

2. Anson, M.L. (1938). The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol*. 22, 79-89.

3. Bascaran, V.; Hardisson, C.; Brana, A.F. (1990). Regulation of extracellular protease production in *Streptomyces clavuligerus. Appl. Microbiol. Biotechnol*. 34, 208-213.

4. Bahrim G.; Scintee (Cotarlet) M.; Negoita T. (2007). Biotechnological conditions of amylase and protease complex production and utilisation involving filamentous bacteria. *The Annals of the University Dunarea de Jos Galati, Fascicle IV-Food Technology, New Series*, Year I (XXX), 76-82.

5. Burg, B. (2003). Extremophiles as a source for novel enzymes. *Curr Opin Microbiol*. 6, 213–218.

6. Chakraborty, S.; Khopade, A.; Kokare, C.; Mahadik, K.; Chopade, B. (2008). Isolation and characterization of novel α-amylase from marine *Streptomyces* sp.D1. J. Molecul Cat B: Enzym. 58, 17-23.

7. Cotarlet, M.; Bahrim, G.; Negoita, T.; Stougaard, P. (2008). Screening of polar streptomycetes able to produce cold-active hydrolytic enzymes using common and chromogenic substrates. *Rom Biotechnol Lett.* 13, 69-80.

8. Cui, L.; Du, G.; Zhang, D.; Chen, J. (2008). Thermal stability and conformational changes of transglutaminase from a newly isolated *Streptomyces hygroscopicus*. Biores. Technol. 99, 3794–3800.

9. De Azeredo, L.A.I.; Castilho, L.R.; Leite, S.G.F.; Coelho, R.R.R.; Freire, D.M.G. (2003). Protease Production by *Streptomyces* sp. Isolated from Brazilian Cerrado Soil. Optimization of Culture Medium Employing Statistical Experimental Design. *Appl Biochem Biotech*.105–108, 749-755.

10. Dastager, S.G.; Lee, J.C.; Ju, Y.J.; Park, D.J.; Kim, C.J. (2008). *Nocardioides halotolerans* sp. nov., isolated from soil on Bigeum Island, Korea. *Syst Appl Microbiol*. 31, 24-29.

11. Dube, M.; Schäfer, C.; Neidhart, S.; Carle, R. (2006). Texturisation and modification of vegetable proteins for food applications using microbial transglutaminase. Springer-Verlag, http://www.aseanfood.info/Articles/11019737.pdf.

12. Fairbairn, D.A.; Priest, F.G.; Stark J.R. (1986). Extracellular amylase sinthesis by *Streptomyces limosus. Enzyme Microb Tech*. 8, 89-92.

13. Feller, G.; Gerday, C. (2003). Psychrophilic enzymes: hot topics in cold adaptation. Nat rev/Microbiol. 1, 200-208.

14. Folin, O. and Ciocalteau, V. (1929). On tyrosine and tryptophane determinations in proteins. *J. Biol. Chem.* 73, 627.

15. Fujiwara S. 2002. Extremophiles: Developments of their special function and potential resources. *J. Biosci Bioeng*. 94, 518-525.

16. Gerday, C.; Aittaleb, M.; Bentahir, M.; Chessa, J.P.; Claverie, P., et al. (2000). Cold-adapted enzymes: from fundamentals to biotechnology.

TIBTECH. 18, 103-107.

17. Ghadin, N.; Zin, N.M.; Sabaratnam, V.; Badya, N. (2008). Izolation and characteriztion of a novel endophytic *Streptomyces* SUK 06 with antimicrobial activity from malaysian plant. *Asian J Plant Sci.* 7, 189-194.

18. Hogue, M.M.; Khaman, M.; Shiekh, M.A. (2006). Characterization and optimization of α-amylase activity of *Streptomyces clavifer*. *Pak. J. Biol. Sci.* 9, 1328-1332.

19. Jorgensen, H.; Fjaervik, E.; Hakvag, S.; Bruheim, P.; Bredholt, H.; Klinkenberg, G.; Ellingsen,T.E.; Zotchev, S.B. (2009). Candicidin biosynthesis gene cluster is widely distributed among *Streptomyces* spp. isolated from the sediments and the neuston layer of the Trondheim fjord, Norway. *Appl Environ Microbiol.* 75, 3296-3303.

20. Kamoun, A.S.; Haddar, A.; Ali, N.E.H; Frikha, B.G; Kanoun, S.; Nasri, M. (2008). Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. *Microbiol. Res.* 163, 299-306.

21. Kim, S.B; Falconer, C.; Williams, E.; Goodfellow, M. (1998). *Streptomyces thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydus* sp. nov.*,* two moderately thermophilic carboxydotrophic species from soil. *Int. J. Syst. Bacteriol.* 48, 59-68.

22. Lane, D.J. (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp 115-175.

23. Lazim, H.; Mankai, H.; Slama, N.; Barkallah, I.; Limam, F. (2009). Production and optimization of thermophilic alkaline protease in solid-state fermentation by *Streptomyces* sp. CN902. *J Ind Microbiol Biotechnol.* 36, 531–537.

24. Macedo, J.A. (2007). Optimization of medium composition for transglutaminase production by a Brazilian soil *Streptomyces* sp.. *Electronic J Biotechnol.* 10:618-626.

25. Madigan, M.; Martinko, O, J. (2005). Brock Biology of Microorganisms, 11 Editions, Publisher Benjamin Cummings.

26. Margesin, R.; Shinner, F. (1999). Biotechnological Applications of Cold-Adapted Organisms, Heidelberg: Springer-Verlag Berlin.

27. Marx, J.C.; Collins, T.; D'Amico, S.; Feller, G.; Gerday, C. (2006). Cold-adapted enzymes from marine Antarctic Microorganisms. *Marin. Biotechnol.*, 293-304.

28. Metha, V.J.; Trumar, J.T.; Singh, S.P. (2006). Production of alkaline protease from an alkalophilic actinomycete. *Biores. Technol.* 97, 1650-1654.

29. Moreira, K.A.; Cavalcanti, M.T.; Duarte, H.S.; Tambourgi, E.B.; Silva, V.L.; Porto, A.L.; Filho, J.L. (2001). Partial characterization of proteases from streptomyces clavuligerus using an inexpensive medium. *Braz. J. Microbiol.* 32, 23-28.

30. Ozer, B.; Kirmaci, H.A.; Oztekin, S.; Hayaloglu, A.; Atamer, M. (2007). Incorporation of microbial transglutaminase into non-fat yogurt production. *Int Dairy J.* 17, 199–207.

31. Park, J.W.; Oh, Y.S.; Lim J.Y; Roh, D.H. (2006). Isolation and Characterization of Cold-adapted strains producing β-galactosidase. *J. Microbiol.* 4, 396-402.

32. Ranganan, S. (1977). Handbook of analysis and quality control for fruit and vegetable products. Second edition. Tata McGram-Hill Publishing Company Limitated, pp 17-18.

33. Rob, A.H. (1992). A perspective on the biotechnological potential of extremophiles. *Trends. Biotechnol.* 10, 395-402.

34. 23. Syed, D.G.; Agasar, D.; Pandey, A. (2009). Production and partial purification of α-amylase from a novel isolate *Streptomyces gulbargensis. J. Ind. Microbiol. Biotechnol.* 36, 189–194.

35. Sivaramakrishnan, S.; Gangadharan, D.; Nampoothiri, M. K.; Soccol, C.R.; Pandey, A. (2006). α-amylases from microbial sources – an overview on recent developments. *Food Technol. Biotechnol.* 44, 173-184.

36. Thumar, J.T.; Singh, S.P. (2007). Secretion of an alkaline protease from a salt- tolerant and alkaliphilic*, Streptomyces clavuligerus* strain mit-1. *Braz. J. Microbiol.* 38, 766-772.

37. Umezawa, Y.; Ohtsuka, T.; Yokoyama, K.; Nio, N. 2002. Comparison of Enzymatic Properties of Microbial Transglutaminase from *Streptomyces* sp. *Food Sci Technol Res.* 8, 113–118.

38. Vonothini, G.; Murugan, M.; Sivakumar, K.; Sudha, S. (2008). Optimization of protease production by an actinomycete strain, PS-18A isolated from an estuarine shrimp pond. *African J. Biotechnol.* 18, 3225-3230.

39. www.biolog.com. Accessed 26 October 2009.

40. Yang, S.S.; Wang, J.Y. (2000). Protease and amylase production of *Streptomyces rimosus* in submerged and solid state cultivations. *Bot. Bull. Acad. Sin.* 40, 259-265.

41. Zhang, Y.; Menga, K.; Wang, Y.; Luoa, H.; Yang, P.; Shi, P.; Wu, N.; Fan, Y.; Li, J.; Yao, B. (2008). A novel proteolysis-resistant lipase from keratinolytic *Streptomyces fradiae* var. k11. *Enzyme Microb. Tech.* 42, 46–352.