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Characterisation of Newly Polar Psychrotrophic Streptomycetes Isolates from Polar Soils with Cold Adapted Bioremediation Potential

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Abstract

Novel polar streptomycetes coded 4 Alga, P2C4 and MIUG 12P, isolated from soil and vegetation samples prelevated from East Antarctica, were genetically and biochemically characterized in order to set up the phylogeny and to identify their capability to grow at lower temperature and to generate cold-active amylase and protease. The 16S rRNA gene sequence of isolate coded 4 Alga was 100% identical to sequences of *Streptomyces* sp. isolates from Norway and from the Solomon Islands, which also were the closest relatives of P2C4 (99.53%) and MIUG 12P (99.53%) isolates. The data emphasised the skills of the strains *Streptomyces* 4 Alga, *Streptomyces* P2C4 and *Streptomyces* MIUG 12P to biosynthesize cold-adapted amylases and proteases suitable in bioconversion processes at low temperature. These results are quite valuable, because only few streptomycetes, particularly the psychrotolerant ones, have so far been explored for their enzymatic potential in cold conditions.

Keywords: 16S rDNA sequence, *Streptomyces* sp., psychrotrophic streptomycetes, cold adapted enzymes, amylase, protease

Introduction

From biodiversity to biotechnological aspects, scientific investigations related to cold-adapted microorganisms remain rather limited and have expanded only in recent years, particularly as the result of the support of the European Union. Cold environments have to be investigates further so as to discover and to characterize new strains which are cultivable or not. Their enzymes, both innovative and invaluable, are key features of their adaptation to these extreme environments and thus should be isolated, cloned and characterized to gain further insight into the strategies of the adaptation to cold, but also to evaluate their biotechnological potential (Marx *et al.,* 2006).

Among the genera of actinomycetes, the genus *Streptomyces* is represented in nature by the largest number of species and varieties, which differ greatly in their morphology, physiology and biochemical activities (Taddei *et al.,* 2006). *Streptomyces* species, Gram-positive soil bacteria with a high GC content (69-78%) (Anderson and Wellington, 2001; Jeffrey, 2008), have the capacity to produce a vast array of secondary metabolites and extracellular proteins. The latter comprise many hydrolytic enzymes such as amylases, proteases and xylanases allowing *Streptomyces* to grow on polymeric substrates (Nguyen *et al.,*1997).

The phylogenetic relationship can be deduced by comparing sequences of certain macromolecules, that form the ribosome, in particular ribosomal RNAs, which are excellent tools for determining the evolutionary relationship. And because all the cells contain ribosomes this molecule can be and has been used to construct a phylogenetic tree of all life forms. Recognition of ribosomal RNA as a tool for constructing pylogenetic relationship was first made by Carl Woese, an American microbiologist. In brief, genes encoding ribosomal RNA from two or more organisms are sequenced and the sequences aligned and inspected, base by base, in a computer. The greater the difference in ribosomal RNA gene sequence between two or more organisms, the greater their evolutionary distance. These distances are than expressed in the form of a phylogenetic tree (Madigan and Martinkb, 2005). While most studies are concerned with the molecular phylogeny of actinomyces, the similar attention has not been granted to their enzymology. However, the ability to produce a variety of enzymes may be an attractive phenomenon in these prokaryotes (Thumar and Singh, 2007). Therefore, the present study aims were to carry out the molecular phylogeny at the three selected streptomycete isolates from the Antarctic biotope and to detect the skills of the isolates to growth at low temperature and to generate cold-adapted hydrolases (amylase and protease).

Materials and methods

Microorganisms

Soil and vegetation were sampled from East Antarctica. The samples were brought back to laboratories in Romania where they were screened for streptomycetes competent to produce cold-active amylase and protease as described by Cotarlet *et al.* (2008). The stock cultures were maintained at 20°C on Gause-agar medium containing (% w/w): 2.0 potato starch, 0.05 K_2HPO_4, 0.05 $MgSO_4$ 7 H_2O, 0.1 KNO_3, 0.05 NaCl, 0.001 $FeSO_4 \cdot 7H_2O$, 2.5 agar, pH 7.2-7.4. New strains coded 4 Alga and P2C4 were isolated from vegetation samples prelevated from Progress Lake 2 area (East Antarctica) during 2008. The strain coded MIUG 12P was isolated from Antarctic soil in the year 2000 and currently belong to the "Dunarea de Jos" University Microorganisms Collection (acronym MIUG).

Chromosomal DNA(chrDNA) extraction

ChrDNA extraction was achieved by using PowerSoil™ DNA Isolation Kit Sample (MO BIO LaboratoriesInc.), sustainable for polar bacteria.

Spectrophotometer analysis of isolated chrDNA

The chrDNA yields, express in ng/µl, were measured with the Nano Drop ND 1000 Spectrophotometer, toward a blank probe (distillate sterile water).

Polymerase Chain Reaction

The 16S rRNA gene was amplified from the chromosomal DNA as a template via the Polymerase Chain Reaction (PCR). The PCR was performed in a 25.0 µl reaction mixture containing 2.5 µl 10X standard reactive buffer (without $MgCl_2$), 1.25 µl $MgCl_2$ (25 mM), 0.25 µl dNTP (25 mM), 0.25 µl f Taq polymerase (5 units/µl) (Ampligon), 1.0 µl F-primer (27 F), (5'-AGAGTTTGATCCTGGCTCAG-3') and 1.0 µl R-primer (1492R), (Lane, 1991) (5'-GGTTACCTTGTTACGACTT-3') and sterile distillate water up to 25.0 µl. Sterile distilled water which substitute template DNA (positive control) was used as negative control. The DNA templates were first subjected to an initial denaturising step for 5 min at 94°C. The 30 cycles consisted of denaturing at 94°C for 30 seconds, annealing at 55°C for 1 min and elongation at 72°C for 1 min before. A final extension at 72°C for 5 min was included.

Agarose gel electrophoresis

To ensure the PCR-reactions on 5.0 µl of each PCR product 1.0 µl Bromophenol Blue (BPB) was added, then the PCR products were visualised on a 0.8% agarose gel. To evaluate the size of the PCR-products, 4.0 µl of 2-log DNA Ladder (New England Biolabs) was loaded on the gel. The gel was run at 50-100V in a TAE buffer (Tris-Acetate-EDTA) until the BPB was ¾ way down the gel. Then the gel was bathed in an Ethidium Bromide solution for 20 min to dye the DNA. Subsequently it was washed in MQ-H_2O for 15 min before being developed and photographed in Gel Doc 2000 from BIO-RAD Laboratories (UK).

Setting Up the TOPO® Cloning Reaction

Setting up the TOPO® Cloning Reaction for transformation into one Shot® TOP10 Chemically Competent *E. coli* was realised according to the Invitrogen protocol.

Transforming One Shot® TOP10 Competent Cells

The transforming reaction was realised according to the manufacturer's instructions (Invitrogen).

Plasmid DNA Purification

The plasmid DNA purification was achieved using QIAprep Spin Miniprep Kit.

Restriction enzyme digestion of DNA

The restriction enzyme digestion of DNA was realised with restriction endonuclease from *Escherichia coli* called EcoRI (this acronym stands for *Escherichia coli*, restriction enzyme I) which has the 5'...G↓AATTC...3'; 3'...CTTAA↑G...5' palindrome as recognize and cleavage site acording to the Invitrogen protocol.

Phylogenetic analysis of the isolates

The single-stranded 16S rRNA gene sequences of the selected polar bacteria were matched with those from a BLAST search of the National Center for Biotechnology database (NCBI) (http://www.ncbi.nlm.nih.gov) (Hobel *et al.,* 2004; Miteva and Brenchley, 2005). Multiple alignments and blocks of conserved nucleotides were constructed with CLC Sequence Viewer 5.12 (free version), (http://www.clcbio.com) (Hobel *et al.,* 2004).

Growth kinetics of streptomycete isolates

Growth kinetics were studied in a submerged cultivation system, using the medium with the following composition (% w/w): soluble starch 2.0, corn steep liquor 1.0, $(NH_4)_2SO_4$ 0.6, $CaCO_3$ 0.8, NaCl 0.5 and soybean oil 0.02 ml, pH 7.0 (Yang and Wang, 1999), via inoculation with 2% spore suspension, for 8 days, at 230 rpm, at different temperatures (10, 20 and 30°C). The biomass accumulation was plotted as OD_{610} versus time.

Amylase and protease assays

Amylase and protease assays in crude extract after biomass separation at 20°C and 6000 rpm for 15 min were determined.

Alpha-amylase assay using an adapted method based on the difference of the hydrolysis products in 0.1 N Lugol solution was determined. One α-amylase unit represents the amount of enzyme which generates a 0.05 decrease in the optical density, for 1 min, measured at OD_{610} nm, of

the colour iodine-starch complex, into a 1% starch solution, at pH 7.0 and 20°C (Bahrim *et al.,* 2007).

Beta-amylase assay using the Merck method was achieved. One β-amylase unit represented the amount of maltose (in mg) produced by one ml crude extract using 1% starch as substrate, at 20°C and pH 7.0 for 1 min. To quantify the maltose, the Shaffer-Somogyi method was used with few modifications (Ranganna, 1977).

Proteolytic assay via the modified Anson method using 2% casein as substrate (Anson, 1938; Cupp-Enyard, 2008) was used. One Anson unit represented 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 at a speed that facilitates release, in one minute, of the hydrolysis products soluble in the trichloroacetic acid; this provides a coloration equivalent, measured at OD_{670} nm, to one μmol of tyrosine, in the presence of the Folin-Ciocalteau reagent by using a tyrosine standard curve over the range 0.02-0.24 μmol/ml (Folin and Ciocalteau, 1929).

Results and discussion

Phylogenetic analysis of the isolated strains

The DNA sequence of the 16S rRNA gene of the isolates coded 4 Alga, P2C4 and MIUG 12P was carried out. The three DNA sequences were very similar with more than 99% identity between the individual sequences. The search for similar sequences in the GenBank/EMBL/DDBJ database and subsequent alignment of the retrieved sequences showed that all three DNA sequences showed high similarity (99-100%) to known 16S rRNA gene squences from *Streptomyces*.

The 16S rRNA gene sequence of the 4 Alga isolate was shown to be 100% identical to sequences of *Streptomyces* sp. isolates from Norway (accession no. EU263063) and from the Solomon Islands (accession no. GQ924533) (Tab. 1). The 16S rRNA gene sequence of the isolate 4 Alga also was the closest relative of the P2C4 (99.53%) and MIUG 12P (99.53%) isolates.

Kinetics of polar streptomycetes growth in cold conditions

The optimal growth temperature for the streptomycetes isolates was achieved in submerged cultivation at 230 rpm, during 8 days at different temperatures (10, 20, 30°C). The cells multiplication and biomass accumulation were revealed measuring the optical density at 610 nm and plotted against time.

The data presented in Fig. 1 showed that the *Streptomyces* MIUG 12P, P2C4 and 4 Alga did not presented a lag phase. Cell multiplication increased in the exponentially growth phase and reached the stationary phase after the 3 days of submerged cultivation (Fig. 1. a, b). These results are in accordance with Thumar and Singh (2007), which

Tab. 1. Similar sequences in the GenBank/EMBL/DDBJ database

		1	2	3	4	5	6	7	8	9	10
Streptomyces sp. 4 Alga	1		99.93	99.93	99.93	99.93	100.00	100.00	99.80	99.80	99.60
EF571003	2	99.93		100.00	100.00	100.00	99.93	99.93	99.87	99.73	99.53
EF571002	3	99.93	100.00		100.00	100.00	99.93	99.93	99.87	99.73	99.53
AF429394	4	99.93	100.00	100.00		100.00	99.93	99.93	00.87	99.73	99.53
EU443837	5	99.93	100.00	100.00	100.00		99.93	99.93	99.87	99.73	99.53
EU263063	6	100.00	99.93	99.93	99.93	99.93		100.00	99.80	99.80	99.60
GQ924533	7	100.00	99.93	99.93	99.93	99.93	100.00		99.80	99.80	99.60
EU263062	8	99.80	99.87	99.87	99.87	99.87	99.80	99.80		99.73	99.53
Streptomyces P2C4	9	99.80	99.73	99.73	99.73	99.73	99.80	99.80	99.73		99.53
Streptomyces MIUG 12P	10	99.60	99.53	99.53	99.53	99.53	99.60	99.60	99.53	99.53	

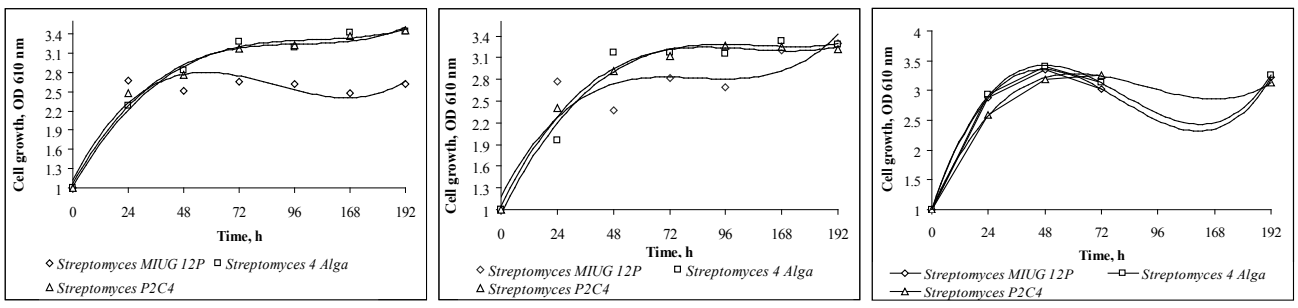

Fig. 1. Growth kinetics of *Streptomyces* 4 Alga, *Streptomyces* P2C4 and *Streptomyces* MIUG 12P in submerged cultivation at 230 rpm; initial p H7.0; inoculum 2% spore suspension; incubation period: 8 days; incubation temp.: 10°C (a), 20°C (b), 30°C (c)

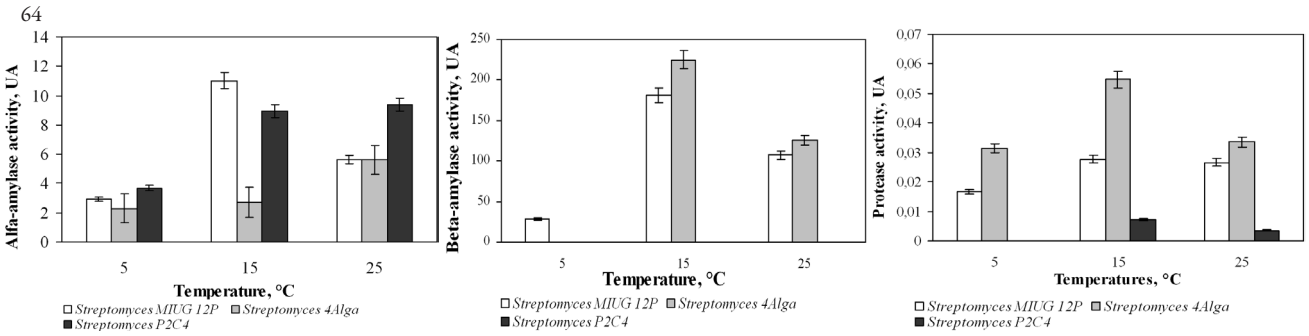

Fig. 2. Amylase and protease activities of crude enzymes extract at lower temperature. The data are the average of two independent assays

argue that *Streptomyces clavuligerus* strain mit-1 reached the stationary phase after 100 h.

At lower temperatures (10, 20°C) it can be assumed that the studied streptomycetes isolates grow proper, the cell density was superior OD_{610}=3.46, after 192 h of submerged cultivation at *Streptomyces* 4 Alga and 3.44 for *Streptomyces* P2C4 (Fig. 1. a, b) at 10°C. Instead, *Streptomyces* MIUG 12P grow proper at 20°C, after 192 h of submerged fermentation (OD_{610}=3.29). Interestingly, these results assumed that studied polar streptomycetes were cold-adapted psychrotrophic strains. In addition, *Streptomyces* 4 Alga was found to be more sensitive to higher temperature than was *Streptomyces* P2C4.

The influence of lower temperature on enzyme activity

The amylase and protease activities analysed in crude extracts at different low temperatures were established (Fig. 2).

Whereas, *Streptomyces* MIUG 12P and *Streptomyces* P2C4 exhibit the highest alpha-amylase activity at 15 and 25°C respectively 11.02 UA and 9.37 UA, *Streptomyces* 4 Alga revealed the highest activity at 25°C but the activity was poorer compared to the other isolates (5.63 UA). The beta-amylase generated by *Streptomyces* MIUG 12P and *Streptomyces* 4 Alga showed the upper limit of activity at 15°C (181.48 UA and 225.28 UA, respectively). Though, considered isolates were competent to biosynthesized cold-active protease, as the maximum yield was registered at 15°C.

The results certify the skills of the *Streptomyces* 4 Alga, *Streptomyces* P2C4 and *Streptomyces* MIUG 12P isolates to produce cold-adapted amylase and protease. These enzymes can be employed in biotechnological processes conduced at low temperatures in order to reduce the risk of contamination by mesophiles and also to save energy (Rob, 1992; Gerday *et al.,* 2000; Feller and Gerday, 2003; Marx *et al.,* 2006; Metha *et al.,* 2006).

Conclusions

The 16S rRNA gene sequence of the polar isolate *Streptomyces* 4 Alga exhibited a 100% match to sequences of *Streptomyces* sp. isolates from Norway and from the Solomon Islands, which were also the closest relatives of the *Streptomyces* P2C4 and *Streptomyces* MIUG 12P polar isolates.

Growth kinetics considered at lower temperature emphasize that polar streptomycetes strains, *Streptomyces* 4 Alga, *S.* P2C4 and *S.* MIUG 12P, were cold-adapted psychrotrophic bacteria.

The strains *Streptomyces* MIUG 12P and *Streptomyces* P2C4 exhibit the highest alpha-amylase activity at 15°C and 25°C. The beta-amylase generated by *Streptomyces* MIUG 12P and *Streptomyces* 4 Alga revealed the maximum yield at 15°C. Thus studied isolates are able to produce cold-adapted protease.

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