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Lipolytic activity of genus *Pseudomonas*

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ABSTRACT

Pseudomonas strains were examined for the production of lipolytic enzymes. Bacteria synthesize extracellular lipase and phospholipase type-C. The majority of the strains of *Pseudomonas sp.* are producers of lipase and phospholipase-C. Phospholipase-C activity is maximal in the initial stationary phase – 12th hour, while the maximum lipase secretion found in the late stationary phase. The production of both enzymes is positively influenced by the addition of extra carbon sources at a concentration of 0.5% for soybean-casein medium that is suitable for cultivation of species *Pseudomonas.*

Key words: lipolytic enzymes, lipase, phospholipase C, genus *Pseudomonas*, bacterial growth

Introduction

Bacteria produce different classes of lipolytic enzymes, including carboxylesterase which hydrolyze ester-containing small molecules, at least partially soluble in water, true lipases that show maximum activity in terms of water-insoluble long-chain triglycerides, and various types of phospholipase (Titball, 1993; Songer, 1997).

Lipase (triacylglycerol acylhydrolase, EU 3.1.1.3) belong to the group of hydrolases which catalyze the hydrolysis of mono-, di- and triacylglycerides to glycerol and fatty acids in the interface water-oil (Brockman, 1984). They perform a wide range of bioconversion reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis, aminolysis (Pandey et al., 1999). Lipases are enzymes which have potential applications in the food, fur, textile, pharmaceutical, cosmetic and paper industry, due to their flexibility (Rajendran et al., 2009).

Many lipases have been isolated from plant, animal and microbial organisms but microbial lipases are those who find the greatest application. Among the producers of bacterial lipases are species of the genera *Pseudomonas, Burkholderia, Vibrio, Acinetobacter, Bacillus, Staphylococcus* (Arpigny & Jaeger, 1999).

Particular attention is focused on specific classes of enzymes of species *Pseudomonas*, that are among the first studied and used in biotechnological production but also because of their involvement in bacterial pathogenesis. Lipases are found in a number of *Pseudomonas* (Junwal et al., 2003; Kim et al., 2005; Noureddini et al., 2005; Karadzic et al., 2006; Singh & Banerjee, 2007; Wang et al., 2009). Enzymes of *P. aeruginosa*, *P. cepacia* and *P. fluorescens* obtained in industrial conditions and are used in organic synthesis, including catalysis of reactions in aqueous solutions (Karadzic et al., 2006; Reetz & Jaeger, 1998).

Species of the genus *Pseudomonas* are active producers of phospholipase-C (Doi & Nojima, 1971; Lysenko, 1973; Sonoki & Ikezawa, 1975; Vasil et al., 1990; Titball, 1993). Some phospholipase have toxic and cytolytic properties and proven involvement in the pathogenesis of various diseases (Coutihno et al., 1988; Meyers & Berk, 1990; Meyers et al., 1992). Furthermore the enzyme of *P. aeruginosa* is one of the toxins that causes destruction of lung surfactant. Phospholipase-C (PLC) facilitates invasion of lung tissues and the development of dermal necrosis (Liu, 1976). All virulent strains of *P. aeruginosa* produce toxin and those of urinary isolates showed the highest activity (Berka et al., 1981).

Phospholipase-C acts on phospholipid cellular membranes and this interaction can be used to study the

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composition of phospholipid membranes and mimic the action of eukaryotic PLC on cell metabolism (Titball, 1993; Ignotz & Honeyman, 2000).

The aim of this study is a research of the production of lipolytic enzymes from non-pathogenic strains of *Pseudomonas sp*.

Materials and Methods

Seventeen strains of the genus *Pseudomonas* from the collection of Department of Biochemistry and Microbiology at Plovdiv University "Paisii Hilendarski" (Bulgaria) were examined.

Lipase and phospholipase activity at the first level were reported on Tributyrin agar (Harrigan & Mc Cance, 1976) and Lecithin agar (Chrisope et al., 1976), respectively.

The presence of lipase, phospholipase C and hydrolysis of various phospholipids were demonstrated by thin-layer chromatography method of Ikezawa et al. (1976). Chromatographic plates were used DC Alufolien Kieselgel 60F 254 (Merck). The separation of the reaction products was carried out by system: chloroform:methanol:water (65:25:4) and petroleum ether:diethyl ether:acetate (60:40:2).

For the production of lipase and phospholipase C, respectively, the strains were cultured of soybean-casein medium (SBC) (BulBio-Sofia), and soybean-casein medium with added extra carbon source (0.5% arabinose, 0.5% rhamnose, 0.5 % xylose, respectively).

Lipase activity was determined using the methods of Beisson et al. (2000) and Westers et al. (2005), where as substrates were used tributyrin (98% pure, Sigma) and p-nitrophenyl butyrate (99% pure, Sigma). One lipase unit is the amount of enzyme that liberates 1 μmol fatty acids for 1 min in standard enzymatic conditions.

The protein content in the culture filtrates was determined by the method of Hatree (1972) with bovine serum albumin (SERVA) as a standard.

Phospholipase activity was reported by LV reaction (Kostadinova, 1991) and the method of Takahashi et al. (1981) with phosphatidylcholine (from egg yolk, 99% pure, Sigma) as a substrate. The reaction mixture contained: 0.2 ml 10 mM phosphatidylcholine, 0.2 ml 0.2 M CH_3COONa-CH_3COOH (pH 6.2) and 0.1 ml enzyme. One unit of phospholipase C hydrolyses 1 μmol substrate in 1 minute under the specific conditions of the enzyme.

Results

Seventeen strains of the genus *Pseudomonas* were analysed for the production of lipolytic enzymes. The strains were isolated from soil. They were non-hemolytic in terms of sheep and human erythrocytes. Strains of *Pseudomonas fluorescens* and *Pseudomonas putida* are typified in Institute of Epidemiology and Microbiology (Moscow).

All of the tested strains *Pseudomonas fluorescens* showed lipase and phospholipase-C activities (Table 1).

Table 1. *Lipolytic activity of strains of Pseudomonas. "+" Zone of 1-2 mm around the colony, "+ +" zone of 3-4 mm; "+ + +" zone of 5-6 m.. The results are averages of three experiments. Activity is presented in U / ml.*

Strains	Lipase activity		Phospholipase-C activity
	Tributyrin agar	Triolein agar	Lecithin agar
Pseudomonas fluorescens	++	+	+
Pseudomonas fluorescens AXL	+++	++	+++
Pseudomonas fluorescens B	++	+	++
Pseudomonas fluorescens 5B	+++	++	++
Pseudomonas fluorescens C	+	+	+
Pseudomonas fluorescens 1D	+++	++	+++
Pseudomonas fluorescens 2D	+++	++	+++
Pseudomonas putida	+	+	+
Pseudomonas putida A	++	+	+
Pseudomonas putida S	+	+	+
Pseudomonas sp. 6A	++	++	+
Pseudomonas sp. 1046	+	++	+
Pseudomonas sp. 1442	+++	+	+
Pseudomonas sp. 3903	+	-	+
Pseudomonas sp. 1133	+	++	+
Pseudomonas sp. 1213	-	++	+
Pseudomonas sp. 1090	+	++	+

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Positive lipase activity we found in *P. putida* and in the strains *Pseudomonas sp*. It was established specificity of the lipolytic substrate in two strains of *Pseudomonas sp*. (*Pseudomonas sp.* 3903 and *Pseudomonas sp.* 1213).

Bacteria whit reported lipase and phospholipase-C activities of agar test are cultivated in different liquid culture medium in order to establish appropriate conditions for the production of enzymes.

The activity of lipase secreted in culture supernatants ranged from 0.9 to 1.7 U/ml. The most active producers are strains *Pseudomonas fluorescens* 5B, *P. fluorescens* 1D and *Pseudomonas sp.* 1442.

Regarding the production of phospholipase-C the most active are the strains *P. fluorescens*. In strain *Pseudomonas sp.* 1442 was determined a low phospholipase secretion, in contrast to the high lipase activity of this strain.

Different nutrient mediums were tested for the production of both lipase and pshospolipase-C and the soybean-casen medium was the most suitable for it. In order to stimulate enzyme production arabinose, xylose, and rhamnose were added to soybean-casein environment, respectively, at a concentration of 0.5%.

Lipase activity of strain *P. fluorescens* 5B increased by 29% (over the main activity of soybean-casein medium accepted as 100%) due to the introduction of xylose in the culture medium. With the addition of rhamnose stimulation was 17% (Figure 1). Lipase secretion of *P. fluorescens* 1D is increased in the presence of xylose by 25%. In strain *Pseudomonas sp.* B, activity is stimulated by 22% by the addition of xylose and arabinose, respectively.

Production of phospholipase-C in *P. fluorescens* 5B affected by xylose and arabinose, respectively 66 and 16%. These carbon sources stimulate phospholipase activity of *P. fluorescens* 1D with 42 and 14%, respectively (Figure 2). In *P. fluorescens* B is reported an increase of 25%, 5% and 15%, respectively from xylose, arabinose and rhamnose.

The protein content in the culture filtrates of *P. fluorescens 5B* and *P. fluorescens* 1D is 9.2 and 9.5 mg/ml protein, respectively.

Strains *P. fuorecsens* 5B and *P. fluoresces* 1D were tested for enzyme production dynamics. The samples were taken at intervals of 4th hour, in which is defined extracellular lipase and PLC activity. The results are presented in Figure 3 and Figure 4.

Secretion of lipase in the culture medium in both strains began on the 4th hour, gradually increasing and reached a peak on the 20th hour. The maximum enzyme activity was reported in the late stationary growth phase of the cultures (Figure 3).

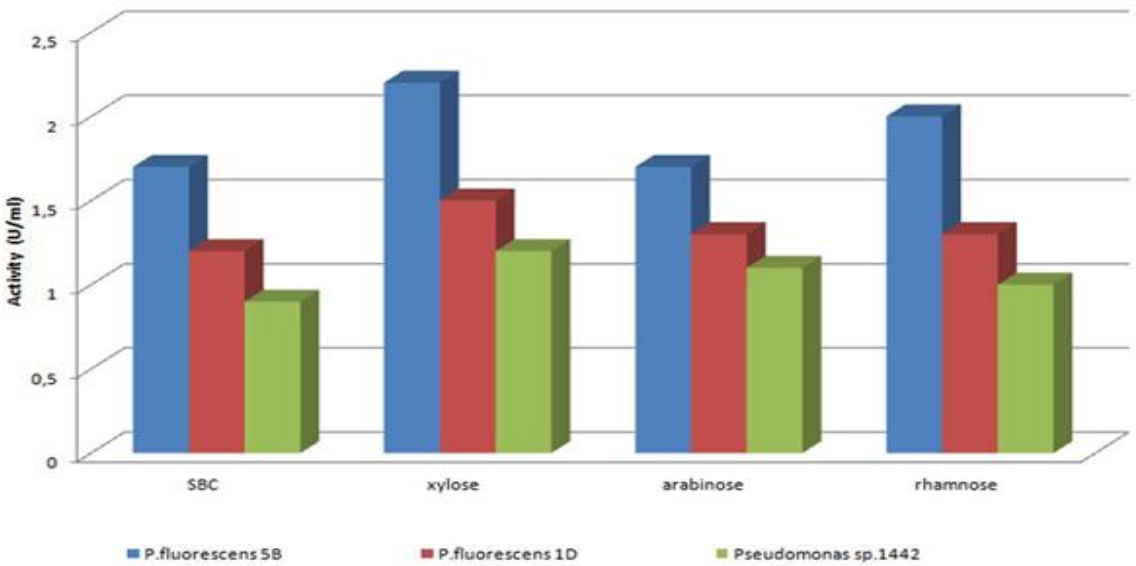

Figure 1. *Effect of extra carbon sources on lipase production of strains of Pseudomonas. Activity is defined substrate p-nitrophenyl butyrate. The results are averages of 5 experiments.*

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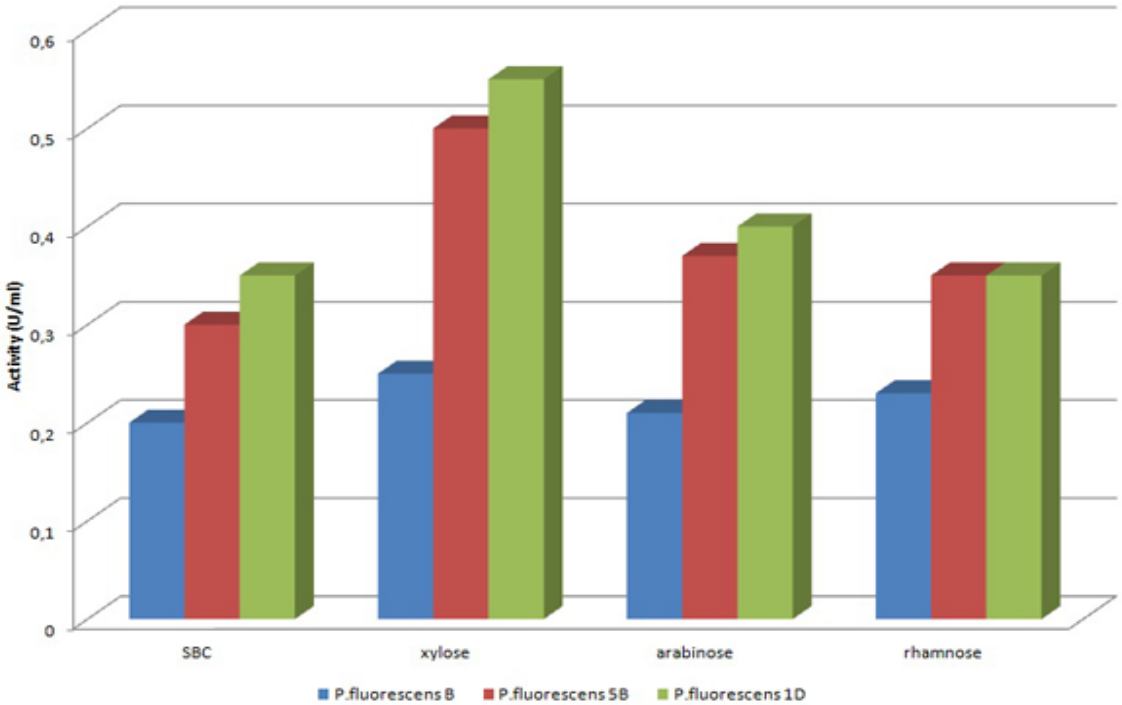

Figure 2. *Secretion of phospholipase-C in the presence of monosaccharides added to the basal medium (soybean-casein) at a concentration of 0.5%. The results are averages of 5 experiments.*

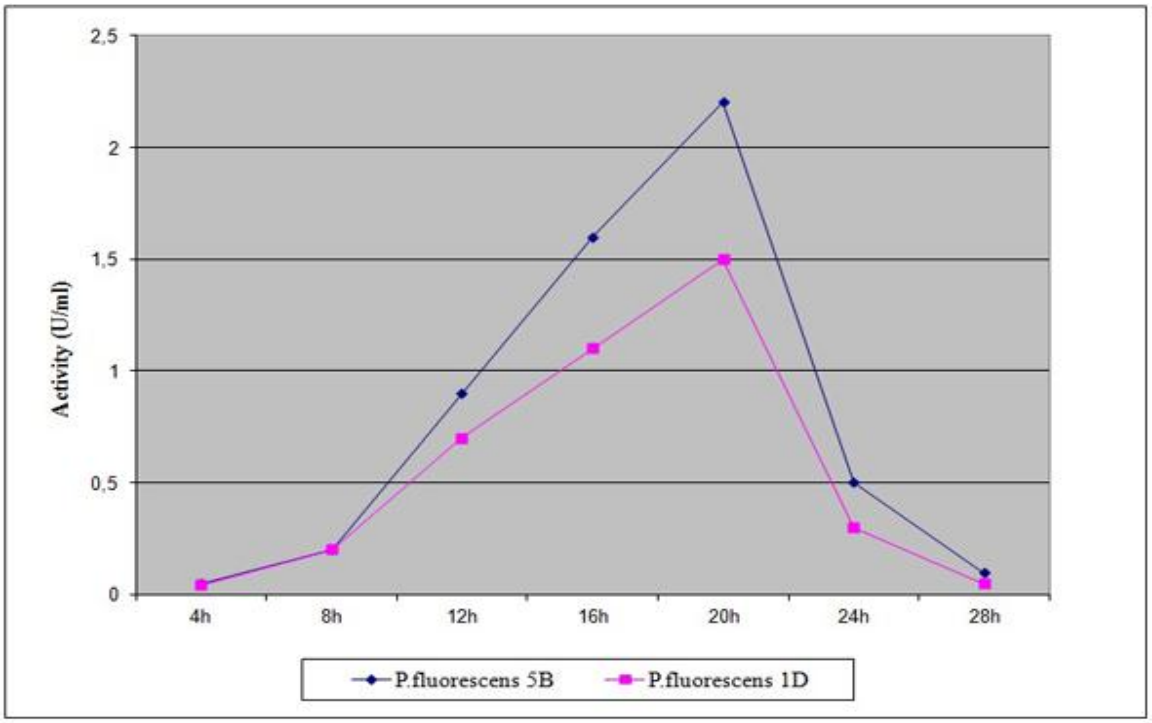

Figure 3. *Production of lipase by P. fluorescens 5B (♦) and P. fluorescens 1D (■). Activity is presented in U/ml in substrate p-nitrophenyl butyrate.*

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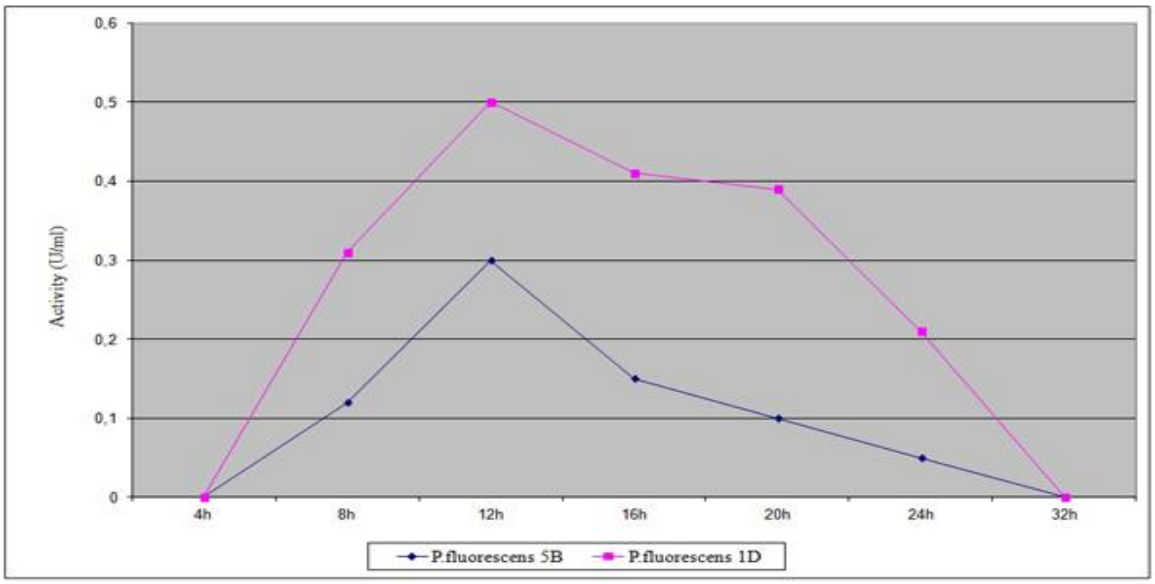

Figure 4. *Extracellular phospholipase activity of strains P. fluorescens 5B (♦) and P. fluorescens 1D (■). Activity is presented in U/ml.*

Production of phospholipase-C in *P. fluorescens* 1D begins on the 4th hour, rising incrementally, reaching a maximum (0.5 U/ml) in the initial stationary phase of growth – 12th hour (Figure 4). After reaching the peak enzyme activity remained relatively high until the 20th hour, which shows good resistance to changes of the enzyme in the culture medium. In *P. fluorescens* 5B dynamics of phospholipase secretion has a similar profile, but reached maximum activity was lower - 0.3 U/ml.

In the culture medium of strains *P. fluorescens* observed changes in pH. In the initial exponential phase into account acidification (pH 6.8) at the 8th hour, then the environment is alkaline (pH 8.3 at 32th hour). The maximum activity for both enzymes in the pH range 7.5 - 8.0.

Discussion

Microorganisms can utilise two major classes lipids triglycerides and phospholipids as a source of carbon and energy (Trichel et al., 2009). Representatives of the genus *Pseudomonas* synthesize several hydrolytic enzymes, such adaptive measures in response to the different states of the environment in terms of nutrient availability (Arpingy & Jaeger, 1999; Preuss et al., 2001). Although the prevailing view of the role of phospholipase C in the nutrition of bacteria, the enzyme is seen as a virulent factor causing a damage and colonization of host tissues, which in turn provides access to new food sources.

Presented our results confirm the literature on the ability to *Pseudomonas species* are used as producers of lipolytic enzymes (Titball, 1993; Crevel et al., 1994; Reetz & Jaeger, 1998; Karadzic et al., 2006). Tested of us two strains *Pseudomonas fluorescens* were selected as potential producers of extracellular phospholipase C and lipase, respectively. The production of both enzymes is optimal soybean-casein medium with xylose added at a concentration of 0.5%. The presence of an additional carbon source in the medium located in an accessible form, causes stimulation of growth and enzyme synthesis. Under these conditions phospholipase activity of the culture medium reached levels of 0.5 U/ml and 2.2 U/ml for lipase. Literature data were confirmed for the conditions of enzyme synthesis in type *Pseudomonas* (Sonoki & Ikezawa, 1975; Ivanov et al., 1996; Kostadinova, 2003).

Maximum enzyme secretion was found in the stationary phase of growth. Both enzymes begin to secrete in the exponential phase, lipase activity slowly increases and reach a maximum in late stationary phase. It is a drop in activity, probably due to proteolysis.

Secretion of phospholipase C increases incrementally and the peak of activity is reported in the initial stationary phase. In strain *P. fluorescens* 1D phospholipase activity remained relatively high for an extended period (6 hours), which would facilitate the work of isolating the enzyme.

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The future research will explore the isolation and purification of both enzymes of phospholipase C and lipase.

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