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KINETIC PARAMETERS OF PROTEASE ACTIVITY OF *PEPTOSTREPTOCOCCUS* SP. AS A TOOL FOR REGULATION OF DAIRY WASTEWATER TREATMENT

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Abstract

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The protein biodegradation is one of rate-limiting processes during dairy wastewater treatment. An opportunity for process acceleration is addition of microbial cultures with high protease activity or addition of pure proteases. In this regard, protein degrading bacteria *Peptostreptococcus* sp. was isolated during an anaerobic wastewater treatment process for dairy industry with the goal of developing an inoculum for bioaugmentation strategy. The goal of this paper was purposely investigation of the key kinetic parameters of the protease activity of *Peptostreptococcus* sp. that are critical for regulation of protein biodegradation in dairy effluent treatment – temperature, pH, substrate concentration, cationic modulators. A batch process, with mineral medium, casein as sole carbon source and *Peptostreptococcus* sp. as microbial biodegradant, was simulated in lab scale.

The obtained results show that protease activity from *Peptostreptococcus* sp. was increased after 72nd hour of the cultivation process and the specific rate of biodegradation was 0.025 $g.l^{-1}.h^{-1}.mg\ Protein^{-1}$. A Michaelis – Menten plot analysis indicated that Km value of protease activity is 0.5 μM and Vmax is 8.624 μM $Tyrosine.min^{-1}.mg^{-1}Protein$. The optimal conditions for protease activity were at temperature 55°C and at pH 8.00. The divalent magnesium ions in concentration 1, 10, 25, 50 and 100 mM were positive modulator of protease activity while divalent calcium ions in these concentrations were inhibitor. The obtained results discovered possibilities for regulation of wastewater treatment process with the key microbial factor *Peptostreptococcus sp*. The further investigations will be directed to elucidate of information how to increase the amount and activity of *Peptostreptococcus sp*. in activated sludge and biofilm in real dairy wastewater treatment technologies.

Key words: casein biodegradation, bioaugmentation, modulation effect, protease kinetics
Abbreviations: OD – optic density; SR – specific rate of protein biodegradation; PRT – protease activity

Introduction

The necessity of improvement in biological wastewater treatment increases the attention to the specialized enzyme techniques. The enzymes have used as: i/ indicators of the activity of biocenoses (biofilms and activated sludge); ii/ indicators of the rate of specific processes which are catalyzed; iii/ bioaugmentative agents for acceleration of rate-limiting processes in wastewater treatment.

The protein biodegradation is one of rate-limiting processes during dairy wastewater treatment. The low rate of protein hydrolysis led to an increase of the rate of protein accumulation on biomass surface. That may limit the transport of the soluble substrates to the biomass and consequently cause the conversion rate in substrates to decrease. An opportunity for process acceleration is the addition of microbial cultures with high protease activity or addition of pure proteases. Several microbial cultures with high protease activity isolated for dairy wastewater treatment are mentioned: *Citrobacter* sp., *Alcaligenes* sp., *Bacillus* sp., *Proteus* sp., *Pseudomonas* sp., *Sporolactobacillus* sp., *Staphylococcus* sp., *Streptococcus* sp. in the scientific literature (Kosseva et

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al., 2003; Rajeshkumar and Jayachandran, 2004; Kosseva et al., 2007). The protein degrading bacteria *Peptostreptococcus* sp. was isolated in our previously investigation during an anaerobic wastewater treatment process for dairy industry with the goal of developing an inoculum for bioaugmentation (Schneider and Topalova, 2008). This culture showed high effectiveness for biodegradation of milk proteins.

Another opportunity for bioaugmentation is the increase of protease activity by addition of some modulators. Most bacterial proteases belong to the metalloproteases. They are the most diverse category of the proteases and require divalent metal ions for their activity (Kim et al., 2005). In this point of view, the divalent ions of zinc, calcium, iron and magnesium play a role in the control of protease activity (Nies, 1999; Kim et al., 2005) and could be used as a modulator in the bioaugmentative strategy.

The goal of this paper was purposely investigation of the key kinetic parameters of the protease activity of *Peptostreptococcus* sp. that are critical for regulation of protein biodegradation in dairy effluent treatment – temperature, pH, substrate concentration, cationic modulators.

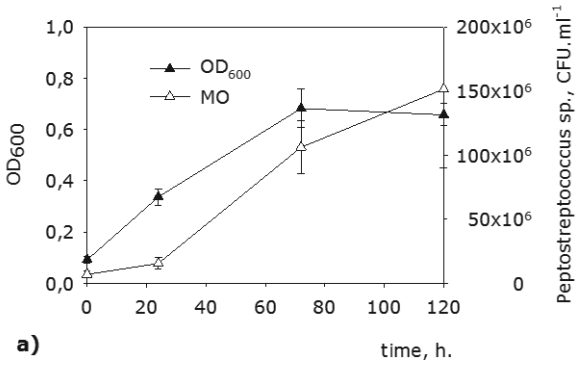
Materials and Methods

A batch process, with mineral medium (Schneider and Topalova, 2008), casein as sole carbon source and *Peptostreptococcus* sp. as microbial biodegradant, was simulated in lab scale. The casein was added as sodium caseinate. The isolation procedure of the pure culture was detailed described in our previous article (Schneider and Topalova, 2008). The cultivation process was performed at 28–30°C for 120 hours, in triplicate.

The biomass, for analysis of Vmax, Km, optimal temperature and pH, modulation effect of calcium and magnesium ions (in concentration 1, 10, 25, 50 and 100 mM) on protease activity, was harvested on 120th hour (Feist and Hegeman, 1969). The calcium and magnesium were added as chlorides. Kinetic parameters Vmax and Km were calculated according Michaelis – Menten plot. The bacterial growth was measured as colony forming units by cultivation on Nutrient Agar and as optical density, measured at 600 nm. The protein concentration was measured according to Kochetov, 1980. The protease activity (PRT) was determined by measuring the release of tyrosine from casein hydrolysis at 500 nm (Ladd and Butler, 1972).

Results and Discussion

Protein biodegradation process with *Peptostreptococcus* sp.: The early phase of the process was established at 24th hour. It was related with low increase of microbial



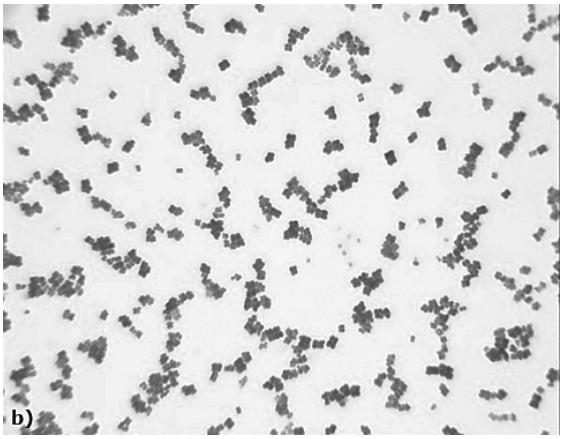


Fig. 1. Dynamics of optic density (OD) and microbial quantity of *Peptostreptococcus* sp. (MO) during biodegradation process (*a*) and (*b*) photograph of microbial cells (LM – 1300X)

biomass (Figure 1a) and low rate of protein biodegradation (Figure 2). The microbial quantity was 2.10^6 CFU.ml^{-1} at this phase. The late phase of the process was ascertained after 72nd hour. It was related with a pick of microbial biomass, a peak of specific rate of protein hydrolysis and a pick of protease activity. The quantity of *Peptostreptococcus* sp. was $1{,}7.10^7$ CFU.ml^{-1} at the end of the cultivation process. The specific protease activity varied between 6 and 10 μM.min^{-1}. mg^{-1}Protein in this phase.

The purity of bacterial culture during the process was investigated by cultivation on Nutrient Agar. The morphology of microbial cells, observed under Light Microscope (1300X magnification) was presented on Figure 1b.

Effect of key factors on protease activity from *Peptostreptococcus* sp.: The effect of temperature in a range 10°C – 70°C on protease activity was estimated at pH 8.0 (Figure 3a). The protease was active at temperature between 30 and 70°C

but inactive at 10 and 20°C. The optimum temperature for this enzyme was ascertained at 55°C. The effect of pH was studied in the range from 6.0 to 11.0. The pH optimum was established at 7.5–8.0 (Figure 3b). On the basis of its optimum pH, this protease would be grouped to the alkaline protease category.

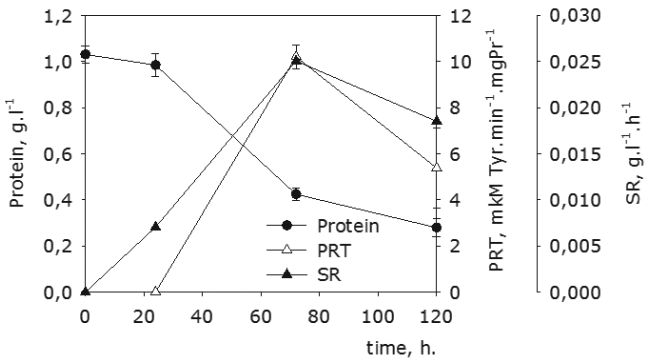
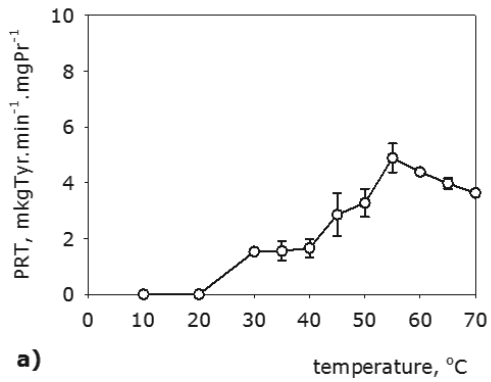


Fig. 2. Dynamics of protein concentration, specific rate of protein biodegradation (SR) and protease activity (PRT) during batch process



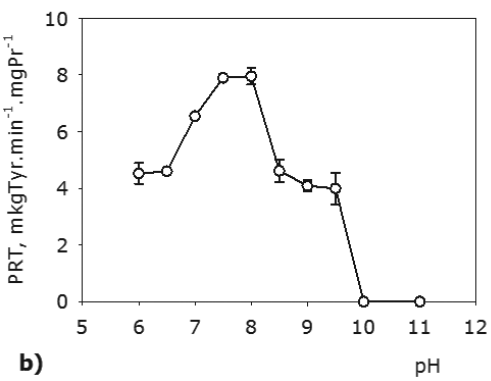


Fig. 3. Effect of temperature (*a*) and pH (*b*) on protease activity from *Peptostreptococcus* sp.

The kinetic parameters of the protease activity were measured in the presence of a range of substrate concentrations from 0.2 to 4 μM. A Michaelis – Menten plot of the data indicated that the Vmax is 8.624 μM tyrosine.min^{-1}.mg^{-1}Protein and Km value is 0.5 μM (Figure 4).

The effect of divalent calcium and magnesium ions on the protease activity was studied and the obtained results are shown in Table 1. Addition of calcium ions in all investigated concentrations inhibited the activity, while the addition of magnesium ions stimulated the enzyme activity. Kim et al., 2002 reported that calcium ions in concentration 1mM stimulated protease activity from the *Bacillus cereus* and

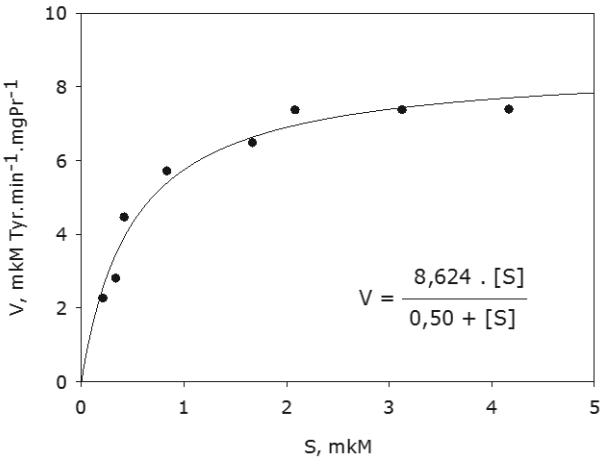


Fig. 4. Kinetic parameters for the hydrolysis of Na-caseinate according to Michaelis-Menten plot (Protease activity was measured at 55°C and pH 8.0)

Table 1

Effect of Ca^{2+} and Mg^{2+} on protease activity from *Peptostreptococcus* sp. The data for control (without modulators) was 100%

Ion	Concentration, mM	Activity, %
No addition	–	100
	1	100
	10	99
Ca^{2+}	25	96
	50	65
	100	20
	1	103
	10	132
Mg^{2+}	25	167
	50	194
	100	104

are essential for enzyme stability. The divalent magnesium ions increased the enzyme activity with 3%, 32%, 67% and 94% in concentration 1, 10, 25 and 50 mM, respectively in comparison with the control without addition of magnesium. However, the concentration of 100 mM magnesium decreased the positive effect on protease activity and it was similar to data, obtained for 1 mM. The obtained results showed that magnesium possessed a capability to protect enzyme against denaturation.

Conclusion

The obtained results indicated that: i/ the optimal conditions for protease activity of *Peptostreptococcus* sp. were at temperature 55°C and at pH 8.00; ii/ the divalent magnesium ions in concentration 1, 10, 25, 50 and 100 mM were positive modulator of protease activity, while divalent calcium ions in these concentrations were inhibitor. These results show that the microbial culture could be applied for the regulation of anaerobic thermophilic wastewater treatment processes in dairy industry. The further investigations will be directed to elucidate of information how to increase the amount and activity of *Peptostreptococcus* sp. in activated sludge and biofilm in real dairy wastewater treatment technologies.

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