AMENDMENT OF THE ACTIVITY OF MICROBIAL PREPARATION LAKTAZYM TO PROTEIN BIODEGRADATION FOR DAIRY WASTEWATER

I. Schneider, Y. Topalova
Sofia University “St. Kliment Ohridski”, Faculty of Biology, Department “General and Applied Hydrobiology”, Sofia, Bulgaria
Correspondence to: Irina Schneider
E-mail: irina_d_s@abv.bg

ABSTRACT
There exist various biological products in the markets for augmentation of wastewater treatment process, but their specificity toward definite substrates is low. In the present study the amendment and the enrichment of biodegradation activity of commercial preparation Laktazym toward a target contaminant in dairy wastewater were investigated. Two microbial dominants – AS-05 and LB-16 with high biodegradation activity to the milk proteins were isolated by the applied selection pressure with casein. Both cultures were detailed characterized by biomass accumulation, effectiveness of organics, protein and lactose removal, as well as by key enzyme activities – total dehydrogenase, phosphatase and nitrate reductase.
Anaerobic biodegradation process for dairy wastewater in a critical situation of destabilized biofilm has been simulated in lab with different biological systems. The experimental data showed greater positive effect on the biodegradation of the organic matter, particularly of the milk proteins for the two modifications of investigated preparation with the selected cultures, than for the variant Laktazym. Moreover at the 48th and the 72nd hour the effectiveness of protein removal for the combination Laktazym + AS-05 (Peptostreptococcus sp.) was higher with 67%, than for the variant only with Laktazym.

Keywords: milk protein, biodegradation activity, dairy wastewater, microbial preparation, anaerobic treatment, destabilized biofilm

Introduction
High levels of proteins with low biodegradation coefficient are containing in dairy wastewater and their biodegradation is a target place for bioaugmentation of water purification process (8, 9). The proteins are an important source of carbon and nitrogen for microbial community but if the accumulation rate is higher than hydrolysis rate, they are adsorbing to biofilm and the rate of biotransformation and biodegradation decreases (3, 8, 12). In the practice, the use of the bioaugmentation strategy as part of water management for the overcome of the critical steps during wastewater treatment process and for the improvement of the overall performance of biological system is wide spread. The enzymes, the pure and mixed microbial population are various commercial biological products with high biodegradation activity but they have low specificity to the target substrate.

The selection of microbial dominants with high specialized, regulated activity towards milk proteins and their inclusion to the preparation Laktazym for amendment of its biodegradation activity to target substrate in dairy wastewater treatment process have been the aim of the present study.

Materials and Methods
Isolation of microbial dominants and selective pressure with casein
In the initial stages of an anaerobic semi-continuous process with model substrate whey, from biofilm were isolated microbial dominants as key physiological groups (aerobic and anaerobic heterotrophs, denitrifying bacteria) microorganisms. In comparative investigation on start-up period of anaerobic filter operation between five different biological systems, the variant of specially adapted activated sludge (AS) enrichment with commercial preparations Laktazym and BiliKuk was most favorable for initial biofilm formation and activation (11). From this biological system and from the variant with specially adapted AS were isolated the microbial dominants.

The selective pressure with Na-caseinate on the seven isolated dominants was accomplished. The casein, the major protein in milk composition (approximately 80% from the total proteins) and in dairy effluents inhibit biological system and the effectiveness of treatment process decreases (8, 12). The two important factors for the selection of pure cultures with high specialized activity towards milk proteins were the growth and casein biodegradation rate. The cell growth by optical density (OD) measurements at a wavelength of 600 nm was monitored. The experiments were performed on a rotary shaker at 200 rpm for 240 hours, in triplicate.

Characterization of the two selected biodegradants
The effectiveness of organics elimination (measured as chemical oxygen demand – COD), protein and lactose removal; the dynamics of the biomass and the activity of the enzymes – total dehydrogenase, phosphatase and nitrate reductase were used for characterization of the two specially selected biodegradants. The inoculation was carry out with 48 hour inoculums in amount 10% v/v, dry weight 6.9 g/l and OD, measured at 600 nm: 0.420 for strain AS-05 and 0.430 for strain LB-16. The cultivation was performed fourfold in
500 ml Erlenmeyer flasks with 350 ml model wastewater on a rotary shaker at 200 rpm for 120 hours.

The taxonomic identification of the two cultures was determined with API test system (API 20A for anaerobes; API 20E for gram – negative rods) and database api web® (BioMerieux Inc.).

Biodegradation process with different biological systems in a critical situation of biofilm destabilization

A comparative study with four biological systems, three variants of commercial preparation Laktazym and the fourth system without microbial preparation (control variant of experiment) was accomplished in anaerobic treatment process for dairy wastewater. The microbial preparation Laktazym (Brave&Brave Ltd.) had stable long-term positive effect on the anaerobic wastewater treatment process (9). This commercial product was selected as a research object for amendment and stimulation of its biodegradation activity toward the milk proteins. The two specially selected dominants for enrichment preparation Laktazym were added to commercial product as cell suspension (10^11 cells/ml). An inoculum in exponential phase was used.

The role of the four biological systems on protein biodegradation was investigated in a critical moment of anaerobic filter operation – destabilized and inhibited biofilm. The simulation of low biofilm activity was achieved by one monthly period, in which the system was not feed with fresh media. It leads to accumulation of harmful metabolites in the system and suppression of the immobilized microorganisms.

Wastewater and bioreactors

A mineral medium with composition (in g/l): NH_4Cl 0.57, KH_2PO_4 0.43, K_2HPO_4 1.09, Na_2HPO_4 1.33, MgSO_4·7H_2O 0.023, CaCl_2 0.028, FeCl_3·6H_2O 0.025 was used in the selection and the cultivation of the biodegradients, as well as in the simulated biodegradation process in a critical situation of destabilized biofilm. In the selective procedure as a sole source of carbon and energy for microbial growth was used Na-caseinate (1.5 g/l), while in the cultivation period and in the simulated biodegradation process was used whey (3.56 g/l). The characteristics of the used synthetic wastewaters in the different processes are given in Table 1. The values are average between three independent measurements.

The simulated anaerobic biodegradation process in a critical situation of destabilized biofilm with the participation of four different biological systems was carried out in 0.5 l reactors, placed in a thermostat at 28-30 °C. The packing media was gravel with size between 8-16 mm with the suppressed biofilm.

Analytical methods, microbial and enzymological analysis

The chemical parameters – pH, COD, total suspended solids (TSS) have been analyzed according to APHA (1). The protein concentration was determined by Microbiuret method (4) and lactose concentration was measured according to Miller (7).

The amount of aerobic heterotrophs, anaerobic heterotrophs and denitifying bacteria was determined after ultra-sonic desorption of biofilm by plate count technique according to the routine practice (5). The heterotrophs were isolated on nutrient agar, and denitrifiers were cultivated on solid media of Giltay with nitrate as a sole source of nitrogen and glucose as a sole source of carbon. The anaerobic heterotrophs and denitrifying bacteria were cultivated in anaerobic jars with Anaerocult A (Merck & Co., Inc.).

Enzyme indicators – total dehydrogenase activity, index of phosphatase activity and nitrate reductase activity were measured according to the methods of Gabbita & Huang (2); Matavuly et al. (6) and Topalova (10). The protein content of the samples was determined by Microbiuret method (4).

The effectiveness was calculated for the total organic content (measured as COD), protein and lactose. The following formula was used:

$$\text{Effectiveness} = \frac{C_t}{C_0} \cdot 100\%,$$

where Co – quantity of investigated parameter at the initial moment; Ct – quantity of investigated parameter at the moment t.

All parameters were measured in triplicate and the data were analyzed using Sigma Plot, Sigma Stat and Excel (Microsoft Corp.). To determine statistical association and coefficient of determination between the total dehydrogenase activity and the effectiveness of protein removal were used Spearman Rank Correlation Coefficient (non-parametric method).

**TABLE 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Isolation of the dominants</th>
<th>Selective pressure with casein</th>
<th>Cultivation process</th>
<th>Biodegradation process</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>-</td>
<td>7.58</td>
<td>7.55</td>
<td>7.76</td>
<td>7.46</td>
</tr>
<tr>
<td>Temperature</td>
<td>°C</td>
<td>28-30</td>
<td>28-30</td>
<td>25-28</td>
<td>28-30</td>
</tr>
<tr>
<td>COD</td>
<td>g/l</td>
<td>3.85</td>
<td>1.90</td>
<td>3.63</td>
<td>3.82</td>
</tr>
<tr>
<td>Protein</td>
<td>g/l</td>
<td>1.01</td>
<td>1.00</td>
<td>0.95</td>
<td>1.03</td>
</tr>
<tr>
<td>Lactose</td>
<td>g/l</td>
<td>2.84</td>
<td>-</td>
<td>2.01</td>
<td>2.24</td>
</tr>
</tbody>
</table>

Key parameters of synthetic dairy wastewater in the different processes
Results and Discussion
The initial investigations were related to selection of pure cultures with high biodegradation activity toward the milk proteins and deeper characterization of the isolates. The amendment of biodegradation activity of a commercial product toward target substrate in dairy wastewater treatment process was investigated by the inclusion of special selected biodegradants to the preparation Laktazym in following stages.

Selection of pure cultures – biodegradants
Isolation of microbiological dominants by key physiological groups (aerobic and anaerobic heterotrophs, denitrifying bacteria)
The detailed microbiological characterization of biofilm, treated dairy wastewater, was carried out for two biological systems - specially adapted activated sludge (AS) and AS enriched with commercial preparations Laktazym and BiliKuk. The aim was preliminary selection of microbial dominants by key physiological groups (aerobic and anaerobic heterotrophs, and denitrifying bacteria). The biodiversity of the two investigated variants by physiological groups is comparatively presented in Fig. 1. The higher morphological biodiversity of the combination AS + Laktazym + BiliKuk than variant with AS suggested more metabolic pathways for utilization of the contaminants in dairy wastewater. Some colonies with very small size were noted as not differentiated (ND). The denitrifying bacteria were the most various, followed by the aerobic and anaerobic heterotrophs. As a result of the lack of strictly anaerobic conditions the three physiological groups overlap in the functions. The lower level of biodiversity in the variant with AS determine higher percent of microbial dominants, while the combination from AS and microbial preparations Laktazym and BiliKuk was presented with lower percent of the dominants.

Metabolic activity toward the model substrate casein
The seven dominants were subjected to selective pressure with Na-caseinate for differentiation of cultures with high

Morphological characterization, growth and casein degradation rates of the dominants

<table>
<thead>
<tr>
<th>Dominant</th>
<th>Morphology</th>
<th>Gram stain</th>
<th>Spores</th>
<th>Rate of growth, h⁻¹ ± SD *</th>
<th>Biodegradation rate of casein, mg/l.h ± SD *</th>
<th>Biodegradation rate of casein, mg/l.h ± SD #</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-02</td>
<td>rod</td>
<td>+</td>
<td>+</td>
<td>0,0003 ± 0,0001</td>
<td>1,28 ± 1,39</td>
<td>1,71 ± 0,19</td>
</tr>
<tr>
<td>AS-05</td>
<td>cocci</td>
<td>+</td>
<td>-</td>
<td>0,0101 ± 0,0024</td>
<td>ND</td>
<td>1,73 ± 1,47</td>
</tr>
<tr>
<td>AS-08</td>
<td>rod</td>
<td>+</td>
<td>+</td>
<td>NG</td>
<td>0,80 ± 0,71</td>
<td>1,11 ± 0,26</td>
</tr>
<tr>
<td>LB-01</td>
<td>cocci</td>
<td>+</td>
<td>+</td>
<td>NG</td>
<td>1,84 ± 0,45</td>
<td>1,33 ± 0,09</td>
</tr>
<tr>
<td>LB-02</td>
<td>cocci</td>
<td>-</td>
<td>-</td>
<td>0,0001 ± 0,0002</td>
<td>0,90 ± 0,36</td>
<td>1,51 ± 0,15</td>
</tr>
<tr>
<td>LB-11</td>
<td>cocci</td>
<td>-</td>
<td>-</td>
<td>0,0003 ± 0,0001</td>
<td>0,97 ± 0,93</td>
<td>1,54</td>
</tr>
<tr>
<td>LB-16</td>
<td>rod</td>
<td>-</td>
<td>-</td>
<td>0,0026 ± 0,0025</td>
<td>3,85 ± 1,08</td>
<td>1,23 ± 0,35</td>
</tr>
</tbody>
</table>

* 120 h.; # 240 h.; NG – no growth; ND – no degradation
proteolytic activity. The follow inclusion of the selected degradants in composition of preparation Laktazym aimed amendment and stimulation of its activity toward more effective elimination of the target substrate in dairy wastewater – the proteins. The morphologic characterization, growth and casein degradation rates of the dominants are given in Table 2. In comparison of the other isolates the cultures AS-05 and LB-16 presented highest growth rate. According to Perle et al. (8) non-acclimatized biomass possessed longer lag-phase (approximately 50 hours), while for investigated non-adapted dominants in this study lag-phase was 24 hours. At the 120th hour from the start of process LB-16 demonstrated the highest degradation rate toward casein, while AS-05 showed highest activity at the 240th hour. The similarity in casein degradation rates and the short lag – phase of the seven isolates confirmed their metabolic activity toward the milk proteins.

Two microbial dominants AS-05 and LB-16 were selected on the base of the growth and on the casein degradation rate. Their inclusion in the preparation Laktazym and their role in the amendment of the biodegradation activity of the preparation toward milk proteins were investigated in the next stages.

Characterization of the two selected biodegradants

The selection of a proper microbial culture for bioamendment of the degradation activity of the autochthonic community toward target contaminants requires an appropriate knowledge on their biodegradation ability. The dynamics of the biomass and the organic removal effectiveness during cultivation period is shown in Fig. 2. In the initial phase of the cultivation (24th hour), the two investigated cultures accumulated similar quantity biomass, while in the end of process (120th hour) AS-05 had higher biomass production. At the 72nd hour of the process the most effective removal of the total organic content (measured as COD) was determined (Fig. 2b). The effectiveness of protein biodegradation for culture LB-16 was highest at the 72nd hour, while for culture AS-05 the effectiveness was maximal at the 120th hour (Fig. 2c). The more accumulated biomass was related to more effective elimination of the proteins.

The low level of organic biodegradation in the initial phase of cultivation was explained with the impossibility of the two selected cultures to degrade lactose. During cultivation process the β-galactosidase, the enzyme that participates in lactose biodegradation, wasn’t expressed. These results were in agreement with the applied selective pressure for the obtaining of cultures with high specialized biodegradation activity toward target contaminant – the proteins.

The results about the investigated enzyme activities during cultivation period are shown in Fig. 3. At the 72nd hour of the process the total dehydrogenase activity (TDA) decreased, while at the 120th hour it was increased (Fig. 3a). This trend was clearly monitored for the culture AS-05 and it was related to more effective biodegradation of the proteins. At the initial and the end phase of the process culture LB-16 presented higher phosphatase activity, while culture AS-05 showed higher activity at the 72nd hour (Fig. 3b). The phosphatase index, another parameter for the total rate of the microbial metabolism, was related with the high effectiveness of organic removal. The nitrate reductase activity at the 72nd hour was highest for both strains (Fig. 3c). The most effective organic removal in this moment led to decrease of the redox-potential and to the utilization of the nitrates as a terminal acceptor of

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**Fig. 2.** Dynamics of the biomass (a), the effectiveness of COD removal (b) and protein removal (e) during the cultivation period for the two selected pure cultures (AS-05 and LB-16)

**Fig. 3.** Dynamics of the activity of the total dehydrogenase - TDA (a), the phosphatase index – PAI (b) and the nitrate reductase – nRA (c) during the cultivation period for the selected dominants AS-05 (●) and LB-16 (○)
Biodegradation process with different biological systems in a critical situation of biofilm destabilization

The bioaugmentation effect of the preparation Laktazym on the activation of the biofilm in a critical situation of destabilization was demonstrated with the specific biological systems. One of all investigated biological systems represented destabilized biofilm and it was a control in the experiment. The amendment of the biodegradation activity of this commercial product toward target substrate in dairy wastewater treatment process was investigated by the inclusion of the selected biodegradants (AS-05 and LB-16) in the preparation Laktazym.

Similar trends about the biomass dynamics during anaerobic biodegradation process for all investigated biological systems were observed (Fig. 4). The parameter total suspended solids (TSS) decreased at the 14th hour and it kept steady to the end of the process. This trend was related to equalization of the detachment rate with the rate of biomass accumulation. The higher content of TSS approximately with 2.5 g/l was observed for the three variants of preparation Laktazym in comparison with control variant. That fact was explained with the addition of new biomass.

One of the target places in the treatment of dairy wastewater is protein elimination. In view of that reason the selection of the dominants was amended toward isolation of cultures with high biodegradation activity to the milk proteins. The results about the effectiveness of protein removal and the total dehydrogenase activity for the four investigated biological systems are presented in Fig. 5. At the initial phases of the process – the 14th and the 48th hour in the control variant was observed protein accumulation because of decreased biodegradation activity of the destabilized biofilm (Fig. 5a). The obtained data confirmed that the adsorption of the protein on the biofilm decreased metabolic exchange between the cells and environment and led to decrease on slowing down of the rate of biodegradation processes (3, 8, 12). In parallel with the control the three variants with preparation Laktazym effectively eliminated the proteins. The biological system Laktazym + AS-05 demonstrated highest level of protein removal (to 30%). The variants with commercial preparation possessed high reactivity and the utilization of the target contaminant began at the 14th hour, while for destabilized biofilm started the protein biodegradation at the 72nd hour of process. In parallel with the effectiveness of the protein removal the dynamics of the total dehydrogenase activity was followed (Fig. 5b). At the 14th hour of the simulated anaerobic process the four investigated biological systems possessed low dehydrogenase activity. With the time the activity of the total dehydrogenase increased and maximal values were achieved at the 48th hour. Highest values of the enzyme activity, especially at the 48th hour were observed for variant Laktazym + AS-05 and it is related to the accumulation of greater quantity active biomass (Fig. 4).

The strength of the statistical association between the total dehydrogenase activity and the effectiveness of protein removal were determined through Spearman Rank Correlation Coefficient (non-parametric method). The coefficients of correlation and determination for all investigated biological systems during the biodegradation process are presented in Table 3. A negative correlation between the effectiveness of protein elimination and the total dehydrogenase activity was observed. The greater probability for correlation between both variables was confirmed from the smaller P-values. The effective removal of proteins at the 14th hour was related to its hydrolyzation. The successful including of the amino acids at the 48th hour in the general metabolic pathways and its biodegradation were confirmed by the high activity of the total dehydrogenase. The effectiveness of protein elimination in the wastewater depended on the total dehydrogenase activity to 90% (R²=0.90). An exception of the rule was the variant Laktazym + AS-05, where the correlation between both parameters was lower (R²= - 0.79) and the percent of determination decreased to 63%. The reason rarely was the specific character of this process – the 14th hour was related to equalization of the detachment rate with the rate of biomass accumulation. The higher content of TSS approximately with 2.5 g/l was observed for the three variants of preparation Laktazym in comparison with control variant. That fact was explained with the addition of new biomass.

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biological system on subcellular, cellular or supracellular level, than the complex character of the investigated system. It was confirmed from the other three investigated systems.

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Laktazym</th>
<th>Laktazym + AS-05</th>
<th>Laktazym + LB-16</th>
</tr>
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<tbody>
<tr>
<td>R</td>
<td>-0.949</td>
<td>-0.949</td>
<td>-0.794</td>
<td>-0.949</td>
</tr>
<tr>
<td>P-value</td>
<td>0.000</td>
<td>0.000</td>
<td>0.006</td>
<td>0.000</td>
</tr>
<tr>
<td>R²</td>
<td>0.901</td>
<td>0.901</td>
<td>0.630</td>
<td>0.901</td>
</tr>
</tbody>
</table>

The following data analysis aimed deeper understanding of the obtained results about the role and the effect of the three biological systems with preparation Laktazym on the water purification process. The values for the effectiveness of organic biodegradation of the variant Laktazym were accepted for 100% and the values of the combinations between preparation and the pure cultures (AS-05; LB-16) were calculated toward the reference value (100%). At the 14th hour of the process more effective elimination of the total organic content for the variant Laktazym + AS-05 in comparison with the other two variants of Laktazym was observed. It was related to the higher biodegradation activity toward the proteins in the initial phase of the process. A strong positive effect on the protein biodegradation for the combination between preparation Laktazym and culture AS-05 was observed. At the 48th and the 72nd hour of the process the effectiveness of the protein removal for the combination Laktazym + AS-05 was higher with 67% in comparison with variant Laktazym. It was a logical consequence from the addition of active microbial dominants with high biodegradation activity toward the proteins, especially toward casein.

A similar effect of the three variants of the preparation Laktazym on the lactose removal was observed. Nevertheless at the 14th and the 48th hour of the process the combinations between preparation and pure culture, especially for variant Laktazym + LB-16 increased the effectiveness of lactose utilization. During the cultivation period both pure cultures can’t degrade the lactose. Their positive effect may be due to the complex synergetic interactions, as well as to the addition of the metabolic pathways of the different populations and/or inclusion to the subsequent stage from the carbohydrate degradation.

The amendment of biodegradation activity of the community toward target contaminant – the milk proteins was successfully attained by both combinations between preparation Laktazym and the pure cultures (AS-05 and LB-16). The investigated strains were non-spore forming (Table 2), while preparation Laktazym included only spore forming bacteria. Furthermore the pure culture AS-05 was isolated from preliminary adapted real activated sludge (11). These microbial cultures enhanced the biodiversity of the commercial product Laktazym and contributed for the synergetic addition of the metabolic pathways of community level.

**Conclusions**

Two microbial dominants – AS-05 and LB-16 with high biodegradation activity toward target contaminant in dairy wastewater – the milk proteins, were isolated after application of selective pressure with Na-caseinate. In comparison with the preparation Laktazym, the addition of the two modifications of preparation with the selected degradants for the activation of the destabilized biofilm showed greater positive effect on the biodegradation of the organic matter, including protein and lactose. At the 48th and the 72nd hour the effectiveness of protein removal for the combination Laktazym + AS-05 (Peptostreptococcus sp.) was higher with 67%, than for the variant only with Laktazym. The enrichment of the preparation Laktazym by purposely selected microbial cultures AS-05 and LB-16 were appropriate, well working approach for the focusing of the preparation action toward high protein biodegradation.

**Acknowledgements**

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