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EXPERIMENTS FOR D-LACTIC ACID PRODUCTION WITH FERMENTATION

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Lactic acid is one of the oldest known chiral chemicals. Its L-enantiomer is widely produced and used both in the nature and in human society. The renaissance of the L-lactic acid production was forced by the biodegradable plastic production on lactic acid basis which produces PLA. This polymer has good biodegradability, but poor mechanic properties, thus its characteristic is under strong improvements. During this investigations it was established that the stereocomplex polymers from L and D enantiomers has higher melting point resulting in broader application. Unfortunately the production of D-lactic acid was not solved since it is not the natural form of lactic acid. For this reason we wanted to examine the possibilities for D-lactic acid fermentation with Lactobacillus coryniformis. We adapted a new technique for cultivation in microtiterplates. First we successfully optimized the media composition in term of carbon source (glucose) and nitrogen source (yeast extract) and obtained an optimal glucose=67.5 g/L and YE=27 g/L concentration by Gauss-surface fitting. Next we noticed (on observation that the final product titer never surpassed 65 g/L but a lot of glucose remained) that this fermentation is under strong product inhibition. We also examined the inhibitory effect of different form of product lactate (i.e. with Na^+, Ca^{2+} and NH_4^+). We obtained that all the examined products cause total inhibition on cell growth at 90–120 g/L concentration but they differ in their effect between 30–60 g/L: the strongest inhibition occurred at NH_4-LA (both 30 and 60 g/L), the Na-LA has lower inhibition range, but its effect is also equal at 30 and 60 g/L and finally Ca-LA showed hardly inhibition at 30 g/L but strong inhibition at 60 g/L

Keywords: D-lactic acid, Lactobacillus coryniformis, product inhibition, microtiter fermentation

Introduction

Lactic acid is one of the basic molecules in chiral chemistry. Four different ligands are connected onto its central carbon atom, of which ligands' stereoscopic orientation results in two isomers, which are of reflected by mirror form of each other. In the nature the presence of the L-form is universal starting from simple organism (like prokaryotes) to higher order organisms (like humans). Furthermore it is also produced industrially and used in large scale mostly by the food industry. The real massproduction demand on lactic acid arised in the '90s, when the price of crude oil dramatic increased, resulting also in the price elevation of oil based polymers, beside the upcoming of their environmental-toxic effect. Although lactic acid can also be produced synthetically (resulting in racemic mixture of the D and L form) in large scale, but the fermentative production has been most widespread since early of the last century. The homopoylmers resulted by the polymerisation of the L-enantiomer is easily biodegradable, but its mechanic properties are disadvantageous from application point of view (brittle and easy to break). The situation was quite the same in case of polymerisation of D-enantiomer. The poor polymer characteristics were investigated with different additives, meanwhile it became clear, that with simultaneous polymerisation of the two isomers at appropriate polymerisation conditions a stereocomplex polymer can be formed, which has higher melting point, thus the application possibilities were widened [1]. However the industrial production of D-lactic acid has not been solved yet, thus the bottleneck of a widely applicable and at the same time easily biodegradable polymer manufacture is the production of the D-lactic acid.

Although in nature the L-form is common, but between microbes it is possible to find also D or racemic (D, L) lactic acid producers. The chirality of the microbiologically produced lactic acid is depending on the stereospecifity of lactate dehydrogenase (LDH, EC 1.1.1.27) of the producer strain.

In our research group fermentative production of lactic acid and related researches have quite long history [3-6], in the frame of which recently we investigate also the technology of a Biorefinery realizing in Hungary. In a biorefinery (that is a complex raw material processing bio-combinate) it would be possible to utilize different non-food grade crops (wheat (also with fusarium infections), corn or sweet sorghum) for both L-lactic acid and D-lactic acid production, which latter has higher price (value) causing benefits for the investment of the biorefinery.

On the basis of these, our goal in the present work was to establish pre-experiments for D-lactic acid fermentation, and to compare these results with the outputs of L-isomer fermentation. During these experiments we adapted a

new high throughput method, which made it possible to carry out simultaneously high number of experiments on microtiterplates (MTP's).

Materials and methods

We used *Lactobacillus coryniformis* DSM20007 strain previously described in the literature [2] as D-lactic acid producer.

For strain maintenance MRS agar slants specially developed for lactobacilli were used. For fermentation we used MRS broth with different yeast extract (YE) and glucose content both in shaking flasks and in round lowwell (24 cells) microtiter plates (MTP). The applied temperature was 37 °C, whereas 150 rpm shaking was used, and the pH was adjusted to 5.8 twice on every day with 12% ammonium-hydroxide with the help of pH-electodes in the case of shaking flasks (from sample) and with pH-indicator strips in case of MTP cells. For shaking MTPs in a rotary shaker incubator we used 'System Duetz' from Enzyscreen, which consisted of a fixing adapter (clamp) and a 'sandwich cover' that is crucial for aerobic fermentation. The latter one has little holes above filter cloth for gas exchange, and on the other hand it can prevent the individual MTP cells from (cross)contaminations [7].

The glucose and lactic acid content of fermentation broths were analysed on BioRad Aminex HPX-87H column at 65 °C with 0.5 ml/min 5 mM H_2SO_4 eluent and RI detection in a Waters Breeze HPLC system.

In case of shaking flasks biomass growth was followed with OD_{600} photometric measurements, while in the MTP's cells we determined cell density with scanning the transparent MTP, and the obtained grayscale images were evaluated with the calibration curve on *Fig. 1.*

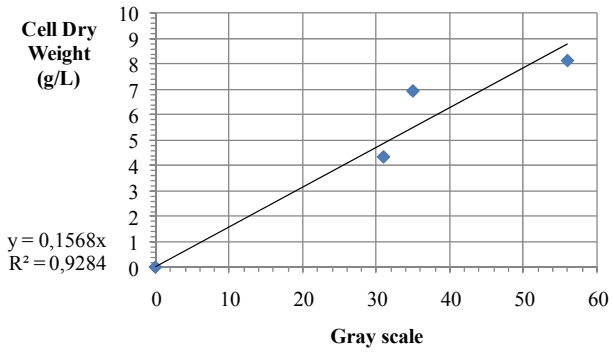

Figure 1: Calibration curve for cell dry weight determination on the basis of grayscale

To obtain the above calibration curve we drove fermentation in shaking flask, and samples was measured both with photometer (OD_{600}), and after placing them into MTP cells with scanner (Epson stylus SX-600FW). For image evaluation we used Bell MicroImage Analyser software, with which we were able to determine the gray grade of the sample between 0 and 256 (0-black, no *Lactobacillus* cells, 256-white, full light reflection).

Results and evaluation

In the first shaking flask experiments the culture grew well beside 20 g/L glucose in 24 h and the decreasing pH also indicated the produced lactic acid. On the basis of this, in the first experiment on MTP we examined the more economical higher substrate concentration (30-60-90-120 g/L), which may cause substrate inhibition or according to the resulted higher amount of lactic acid product inhibition can also occur. Each different substrate concentration was tested with different YE (i.e. nitrogensource) supplementations (5-15-25-35 g/L) which resulted 16 experimental set up. Beside these we designed a central point with 75 g/L glucose and 20 g/L YE running with three repeats and furthermore three "central point's" were also created, but not inoculated to test (cross)contamination.

To evaluate the obtained results we defined a combined parameter (objective function) (Q) to find the optimal media composition with smaller amount of remaining glucose and higher amount of lactic acid yield simultaneously. This was necessary, since the final lactic acid titer could be correlated with the different starting amount of substrate making impossible to compare them. The obtained $Q = Y_{LA}/Glu_{remained}$ values are presented on *Fig. 2* as the function of glucose and YE concentrations.

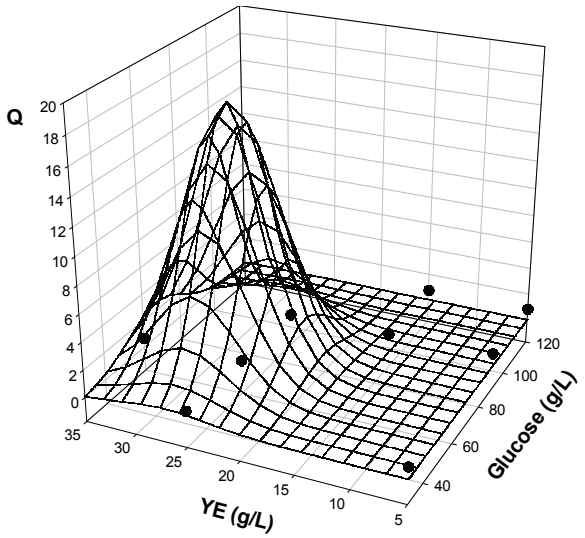

Figure 2: Examination of the media composition on the effectiveness of lactic acid fermentation

Onto the calculated Q values obtained from the experiment we fitted a Gauss-surface (R^2=0.98) with *Sigma Plot 2001* software, and from the fitted constants of the function the location of the maximal Q can be established as follows: Glucose = 67.5 g/L and YE = 27 g/L.

It have to be remarked, that we never found higher lactic acid concentration than 65 g/L, although the obtained more than 90% product yield at lower substrate concentration (for example 30 g/L) predicted very high lactic acid titer at 90 or 120 g/L starting glucose concentration. The problem is clearly demonstrated on *Fig. 3*, indicating product inhibition, since the final glucose remains around zero up to ca 60 g/L of final LA

titer, but above it the final remaining glucose concentration breaks out.

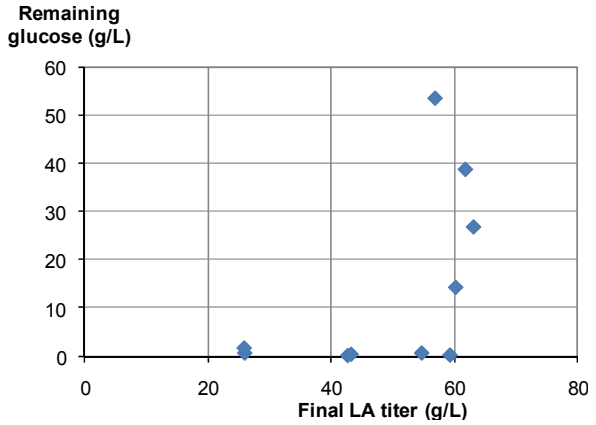

Figure 3: Coming up the question of product inhibition

To examine the product inhibition more detailed a new MTP experiment was designed as follows: 30-60-90-120 g/L initial concentration of different (Na^+, Ca^{2+}, NH_4^+) lactates were applied before inoculation with two replicates of every experiental set up. Becauuse in the existing LA technologies these alkali (NaOH, NH_4OH, $CaCO_3$) are applied for pH controlling during fermentation, we wanted to examine the effects of these. In the cases of 90 and 120 g/L initial lactates we never found any cell growth, indicating full product inhibition. The 30 and 60 g/L cases can be compared on *Fig. 4.*

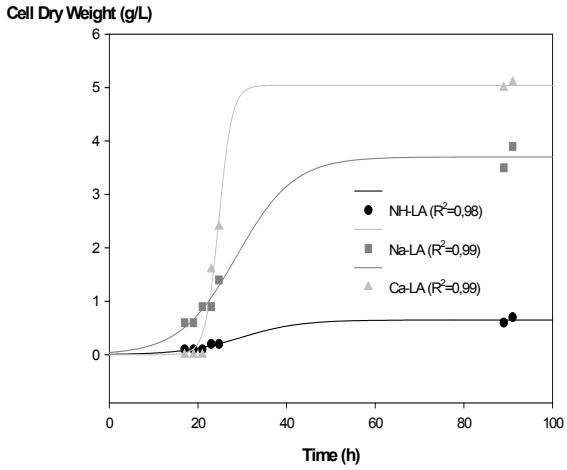

Figure 4: Comparing the different kind of 30 g/L initial lactates

If the two profiles on *Fig.* 4 and *5.* are compared it can be obtained, that ammonium-lactate (NH-LA) has the strongest inhibitory effect and there is no difference between the levels of 30 and 60 g/L, thus already 30 g/L can dramatic decrease in the cell growth of *L. coryniformis*. The inhibitory effect of sodium-lactate (Na-LA) was lower, but also the same at 30 and 60 g/L concentrations. Finally, Ca-LA can hardly inhibit in 30 g/L amount, but it has strong inhibitory effect at 60 g/L concentration.

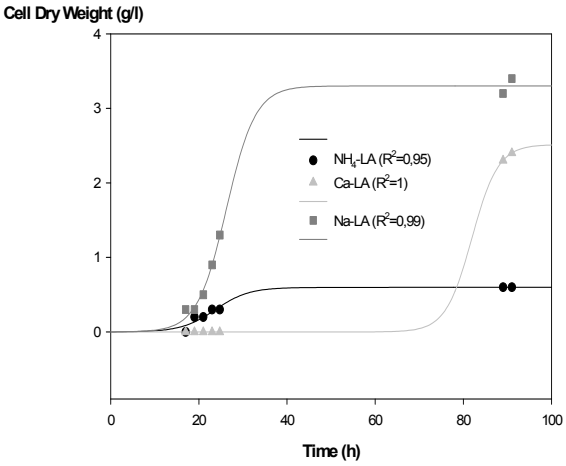

Figure 5: Comparing the different kind of 60 g/L initial lactates

Conclusions

Our preliminary results clearly show, that the lactic acid fermentation of *L. coryniformis* has high yield (>90 %) when applying lower substrate concentration, but the industrially acceptable level of substrate causes product inhibition. On the basis of our experiments we can start to build kinetic approaches to obtain inhibition constants in order to describe the lactic acid fermentation performance with this strain. However, most probably to introduce it to an industrial technology it needs thorough strain improvements.

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