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EXAMINATION OF MEDIUM SUPPLEMENTATION FOR LACTIC ACID FERMENTATION

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Batch fermentation experiments were performed to evaluate the potentials of different fractions of wheat as alternative carbon and nitrogen source during the economical production of lactic acid by a homofermentative mesophilic bacterium. Hydrolysing the starch content of wheat results in well consumable glucose solution, and simultaneously hydrolysing the insoluble protein content (gluten) of wheat the nitrogen source can be assured as well. The necessary yeast extract concentration was 30 g l^{-1} on hydrolysed wheat starch solution without gluten fraction, and it resulted in 2.01 g l^{-1} h^{-1} volumetric lactic acid productivity. Other possible (and usually applied) supplementations (corn steep liquor, yeast autolysate) are neither available in Hungary nor they are economically considerable alternatives. Using the gluten fraction can substitute the major part of the added yeast extract as nitrogen source, but since there is a need for other microcomponents (vitamins, amino acids etc.) of yeast extract, thus a minimal amount of yeast extract is necessary for lactic acid fermentation. The optimized gluten containing medium resulted 2.31 g l^{-1} h^{-1} productivity which is an industrially acceptable result, showing to be an effective and alternative nitrogen source.

Keywords: Lactic acid; Wheat; Hydrolysis; Yeast extract; Gluten.

Introduction

In our days a significantly increased interest became noticeable in the recovery of fermentation products, such as organic acids, feed or food additives and industrial chemicals. Fermentation moves into lower-value higher-volume chemicals, so it becomes necessary to maximize efficiency and minimize costs and waste by-products to compete against traditional alternatives [1].

The use of excess biomass or wastes from agriculture to produce energy, feed or food, and other useful products can be the solution of many economical and ecological problems. Lactic acid can be easily produced by fermentation from different raw materials, applying various technological ways and it can be an appropriate starting-point for several compounds.

The agro-industrial residues (such as the residues of corn, wheat, sweet sorghum or whey etc.) can all be suitable raw materials in the production of lactic acid, and with a well-chosen technology they can satisfy every need of lactic acid bacteria without any or with minimal amount of supplementation.

In Hungary has shown up a claim to use surplus grain capacity (corn, wheat) in green-industrial technologies, and in parallel with oil-processing a new concept was born called "bio-refinery" [2]. The target of this theory is to produce lactic acid from wheat utilizing the whole wheat grain. While the starch content of the wheat serves as carbon source for lactic acid bacteria (after starch hydrolysis forming glucose), the other components (gluten, bran etc.) are also usable by-products of the technology. They can be used for either in production of bio-gas or for direct commercialization. The lactic acid as main product then can be converted in some simple synthetic steps to other chemicals such as poly-lactate (PLA), butyl-lactate, ethyl-lactate, propylene-glycol etc.

Lactic acid bacteria can utilize the hydrolysate of wheat starch [3-6], but these bacteria require a high level of nutrient supplementation including nitrogen source, amino acids, vitamins and microelements [3, 7-15]. To cover these needs generally yeast extract is added to the media as best nutrient source. However the use of yeast extract as only nitrogen and additional nutrient source makes the technology extremely costly. Corn steep liquor (CSL) as supplementation suits to the "bio-refinery concept" and applying it in a previously optimized medium with glucose carbon source and a minimal yeast extract supplementation we achieved an industrially reasonable lactic acid productivity (3.86 g l^{-1} h^{-1}) [16].

While yeast autolysate, peptone, trypton etc. may also come into question as alternative organic nitrogen sources, ammonium-sulphate, ammonium-phosphate can be used as inorganic compounds. After a proteolytic digestion the protein fraction (gluten) of wheat can also

be utilized for fermentation purposes. The proteolysis of gluten can be carried out either separately or simultaneously with the starch hydrolysis [1], depending on the type and the pH optimum of the protease (neutral or alkaline protease). Wheat gluten hydrolysis results in peptide mixtures with high solubility [17-18] helping the microbes to utilize gluten fraction and ameliorating the rheological properties of the medium.

The role of vitamin supplementation in lactic acid fermentation is described widespread but it is specific for the producer strain [19-20]. Since the vitamin need of our bacteria was not described yet, we performed some experiments to test several vitamins.

In this study we report some medium optimization steps for the replacement of expensive nutrient supplementations. Utilization of starch content of wheat as carbon source and the protein content as nitrogen source is reported earlier [3-6] and it may prove a great advantage toward a more economical lactic acid production process.

Materials and methods

1. Microorganism

Lactobacillus sp. MKT-LC878, a facultative anaerobic homofermentative L-lactic acid producer, was obtained from an earlier strain selection program in our research group. The strain was stored on MRS medium agar slants (Difco, USA) at 4 °C.

2. Culture conditions

Precultures for experiments in fermentors were prepared by transferring a stock culture onto two or four slants of MRS agar and incubated at 37 °C for 24 h. Cells were harvested in sterile water and the cell suspension was transferred by a sterile syringe into the bioreactor. For shaking flask experiments inoculation was done by loop. These experiments were carried out in 250-ml flasks containing 100 ml medium. A 2 liter (B. Braun Biostat® M 1800/2000 ml) and four 1 liter (B. Braun Biostat® Q 800/1000 ml) were employed for fermenter operations.

In shaking flask experiments agitation speed and culture temperature were controlled at 200 rpm and 37 °C (Medicor BRI-1 rotatory shaker), and pH was maintained by addition of $CaCO_3$ (stoechiometrically). In the fermentors agitation speed and culture temperature were controlled at 500 rpm and 37 °C respectively, the pH was regulated at 5.8 by 25 % H_2SO_4 and 25 % NH_4OH.

The flasks, the buffering $CaCO_3$ and the supplementing media-components were sterilized in an autoclave at 121 °C for 20 min. The wheat hydrolysate did not need sterilization (because of the applied high hydrolysis temperature) while the vitamin solutions were sterile-filtered.

3. Media and hydrolysis

Basic wheat flour medium I. (without gluten): after adding 83 ml tap water to 100 g wheat flour (type 550), 1 hour kneading and addition of 104 ml tap water and 8.2 µl Shearzyme® 500L enzyme (Novozymes, Denmark) was carried out for the agglomeration process. Gluten fraction was separated by centrifugation (30 min, 3000 rpm, Janetzky K70 D centrifuge), followed by washing with water, and diluting the starch suspension up to 500 ml. The liquefaction of starch was carried out for 40 min by 28 µl Termamyl® SC (α-amylase by Novozymes), at 85 °C and pH 5.5. For the saccharification (separately and prior to fermentation) 80 µl SAN® Super 240 L (glucoamylase and protease by Novozymes), for 46 hours, at 55 °C and pH 5.5 was used. Hydrolysis was performed in a 2 liter B. Braun Biostat® M fermentor.

Basic wheat flour medium II. (with gluten): 100 g wheat flour (type 550, protein content 11% [21], wet gluten content 27%) was suspended in tap water to a final volume of 500 ml. The liquefaction and saccharification steps corresponded to the above mentioned process. Since the SAN® Super 240 L product of Novozyme contains proteases as well, the hydrolysis of gluten was done in line with saccharification.

The additional supplements, i.e. yeast extract (Reanal Budapest, Hungary), corn steep liquor (Hungrana, Szabadegyháza, Hungary), yeast autolysate were added to the basic media before sterilization in shaking flask experiments or before hydrolysis in case of bioreactors. Whey permeate (Friesland-Danone, Nagybánhegyes, Hungary) was used in place of tap water. Yeast autolysate was made from 50 g baker's yeast (Lesaffre, Budapest, Hungary) by adding one drop of toluene, which helps the disintegration of cell wall and the auto-proteolitic activity of yeast cells.

The vitamins (biotin, choline, cyanocobalamine, folic acid, inositol, nicotinic acid, PABA, panthothenic acid, pyridoxine, riboflavin, thiamine and thymidine) were purchased from Sigma-Aldrich.

4. Analyses

Substrates and products were analyzed by HPLC (Waters Breeze HPLC System, BioRad Aminex HPX-87H column on 65 °C, mobile phase: 5 mM H_2SO_4 at flow rate of 0.5 ml min^{-1}).

Cell growth was measured as optical density (Pharmacia LKB-Ultrospec Plus Spektrophotometer) at a wavelength of 600 nm. In the case of shaking flasks the samples had to be acidified to dissolve $CaCO_3$ but it resulted precipitation of wheat proteins which disturbed the optical density measurements.

Results

1. Fermentation on wheat starch hydrolysate without gluten and supplementations

After the hydrolysis of the starch content of wheat flour the carbon source was the glucose content, the nitrogen source was only the water soluble protein content (in case of wheat flour type 550 the water soluble protein content is ~2.5% [21]). There was no further supplementation, the necessary minerals supposed to be stemmed from wheat and from tap water.

The conversion was slow *(Fig. 1)* and after one week cultivation the broth still contained 60 g l^{-1} residual glucose. This indicated the need of more supplementation for the growth of bacteria.

Obviously the obtained lactate yield of 64% and the volumetric productivity of 0.22 g l^{-1} h^{-1} can not be considered as industrially acceptable.

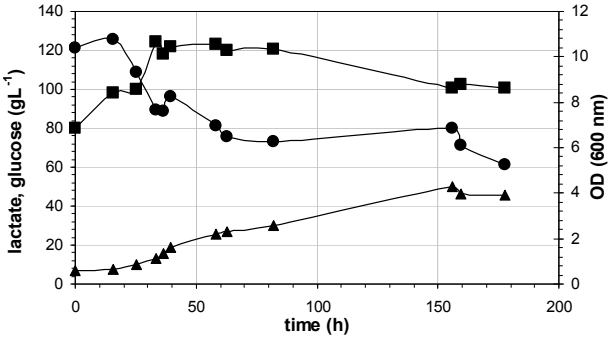

Figure 1: Lactic acid fermentation on wheat starch hydrolysate without gluten and other supplements: (●) glucose; (▲) lactic acid; (■) optical (biomass)density

2. Optimization of wheat starch hydrolysate based medium

To ameliorate the efficiency of fermentation we had to improve the medium with some additional compounds. As a first step the nitrogen supplementation need was examined with three different nitrogen sources: yeast extract (YE), corn step liquor (CSL) and yeast autolysate (Y/t).

The use of yeast extract (rich in vitamins, minerals, amino acids and other easily consumable nitrogen sources) in larger scale makes the process very costly.

Corn steep liquor, the by-product of corn starch producing technology which contains several vitamins and divers nitrogen sources has much lower price, since it is an agro-industrial by-product, thus it has no significant effect on process cost.

Yeast autolysate is made from toluene treated baker's yeast and contains the same nutrient sources as yeast extract, but its prime cost is much lower.

The main goal of these experiments was to find the appropriate amount of every single nitrogen source, applying them with equivalent total nitrogen amount and the results are shown in *Table 1.*

Table 1: The effect of nitrogen-supplementation quality and quantity on lactic acid productivity

N-supplementation	Amount of supplementation (g l^{-1})	Total N-content (g l^{-1})	Productivity (48 h) (g l^{-1} h^{-1})
Yeast extract	10	1	1.71
	20	2	1.86
	30	3	2.01
Corn steep liquor	26	1	1.71
	52	2	1.97
	78	3	1.91
Yeast autolysate	48	1	1.13
	96	2	1.69
	144	3	1.47
No suppl. (control)	0	0	0.01

As shown by the results the yeast extract and the corn steep liquor proved to be the most appropriate. However, the yeast extract in this amount (in a minimal amount of 20 g l^{-1}) makes the lactic acid production significantly uneconomical, while the corn steep liquor is nowadays not available in Hungary. The required total nitrogen amount is 2 g l^{-1} in the case of yeast autolysate, i.e. 96 g l^{-1} yeast autolysate is necessary for the fermentation.

Lab fermentation results with this optimized (yeast autolysate containing) medium *(Fig. 2)* are convincing (lactic acid yield of 93%, productivity of 1.48 g l^{-1} h^{-1}). Nevertheless the enormous (96 g l^{-1}) amount of the required yeast and the complicated previously necessary yeast cell inactivation and for their alcohol producing enzyme system remains partly active even after the inactivation, makes this supplementation inconvenient for a feasible technology.

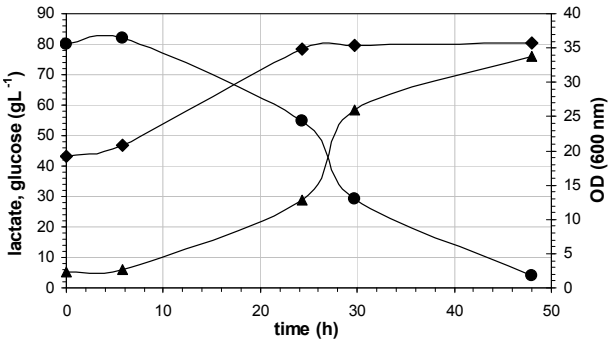

Figure 2: Lactic acid fermentation on optimized wheat starch hydrolysate without gluten: (●) glucose; (▲) lactic acid; (■) optical density

3. Fermentation on wheat flour hydrolysate with gluten

In the question of supplementations the necessity of finding cheap alternatives for yeast extract available in large amounts led to use the whole protein content of wheat (11% [21]). The gluten is the insoluble protein mixture of wheat, thus is not directly usable as medium component. After a proteolytic digestion it can be made water soluble and assimilable for the lactic acid bacteria as nitrogen source.

The proteolysis can be performed simultaneously with the starch hydrolysis and thus the whole flour suspension become usable as fermentation medium.

The considerably long fermentation time beside of significant residual glucose concentration *(Fig. 3)* posed the lack or limitation of nitrogen source or some other nutrients (amino acids, vitamins), which can be overcome by yeast extract.

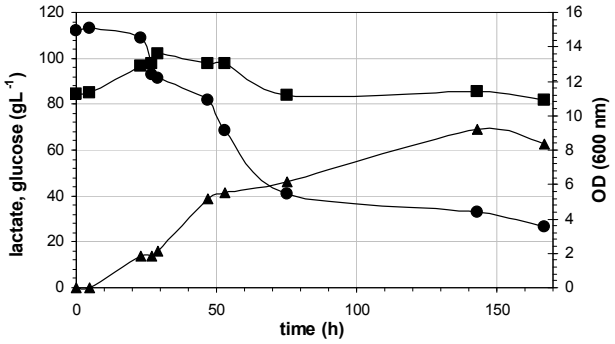

Figure 3: Lactic acid fermentation on wheat flour hydrolysate with gluten:
(●) glucose; (▲) lactic acid; (■) optical density

4. Further experiments to supply the wheat flour based medium

To find an appropriate supplementation supporting lactic acid fermentation on wheat flour based medium II. we tried to replace tap water by whey permeate, the by-product of cheese manufacturing. Since it contains lactose and milk proteins, whey can play the role of carbon and nitrogen source simultaneously. However the fermentation results showed that the whey protein was not sufficient to consume the whole glucose and the lactose content of the medium. Nevertheless the combined lactic acid yield for both carbon sources was 96%, while the half of the lactose content remained in the system and the lactic acid productivity was as low as 0.52 g l^{-1} h^{-1}.

Because the total nitrogen content of wheat flour should cover the nitrogen need of our bacteria, the bottleneck of the fermentation is not the amount of nitrogen source. Since the best proven supplementation yeast extract contains a lot of vitamins beside its proteins as nitrogen source, we performed shaking flask experiments to determine the essential vitamins. *Table 2* contains the applied vitamins and their amounts equivalent to the vitamin content of 20 g l^{-1} yeast extract. In the first step a medium containing the listed 12 vitamins with no other supplementation was applied but the resulted 0.67 g l^{-1} h^{-1} productivity suggested the need of further supplementations. Applying 12 vitamins in a flask together with 2 g l^{-1} yeast extract and the same without vitamins did not show significant difference resulting in 1.19 g l^{-1} h^{-1} and 1.16 g l^{-1} h^{-1} productivity respectively. However these results were convincing that only a minimal yeast extract supplementation could be sufficient to complete sugar-lactic acid bioconversion.

Table 2: Vitamin content of shaking flasks

Vitamin	**Amount of vitamin (µg/100 ml)**
Biotin	6.6
Choline	60
Cyanocobalamine	0.2
Folic acid	3
Inositol	2800
Nicotinic acid	1195.8
PABA	1526
Panthothenic acid	574.4
Pyridoxine	86.4
Riboflavin	233
Thiamine	1059.8
Thymidin	35

5. Optimization of wheat flour hydrolysate based medium (with gluten)

To find the above mentioned minimum amount of yeast extract (as the source of nitrogen or another necessary component) needed to the complete bioconversion of the glucose, the medium was supplemented by YE in different concentrations *(Table 3).*

Table 3: The effect of yeast extract supplementation on lactic acid productivity*

N-supplementation	**Amount of supplementation (g l^{-1})**	**Total N-content (g l^{-1})**	**Productivity (48 h) (g l^{-1} h^{-1})**
Yeast extract	0	0	0.80
	1	0.1	2.23
	2	0.2	2.45
	3	0.3	2.47
	4	0.4	2.29
	5	0.5	2.48
	10	1	2.63
	15	1.5	2.49
	20	2	2.41

*Productivity results were calculated at zero residual sugar concentration

According to the results shown in Table 3 even the use of 1 g l^{-1} yeast extract can be sufficient to complete lactic acid fermentation with the gluten content of the wheat flour.

The lab scale fermentation experiment on this optimized wheat flour based medium (with 1 g l^{-1} YE) reproduced the convincing results of shaking flask experiment with a yield of 90% and a productivity of 2.31 g l^{-1} h^{-1} *(Fig. 4).*

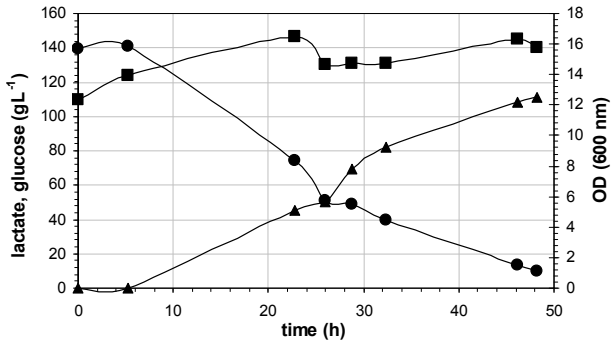

Figure 4: Lactic acid fermentation on optimized wheat flour hydrolysate with gluten:
(●) glucose; (▲) lactic acid; (■) optical density

Conclusion

The wheat contains carbon and nitrogen source in sufficient amount for lactic acid bacteria but in the form of heterogeneous mixture of various starch and protein macromolecules. The hydrolysis of starch and wheat proteins by commercial enzyme products obviously covers the nutrient needs of bacteria. The use of wheat gluten as nitrogen source significantly reduces the need of yeast extract which otherwise would make the lactic acid fermentation very costly. On the basis of a systematic culture medium optimization the initial 20 g l^{-1} yeast extract demand successfully decreased to as low as 1 g l^{-1} and by this process the volumetric productivity of lactic acid increased from 1.86 g l^{-1} h^{-1} to 2.31 g l^{-1} h^{-1} respectively. Hereby we approached the lactic acid fementation efficiency had been reached with a previously optimized medium (3.86 g l^{-1} h^{-1}).

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