First Steps in the Development of a Wheat Flour Based Lactic Acid Fermentation Technology. Culture Medium Optimization

K. Hetényi, Á. Németh, and B. Sevella*
Budapest University of Technology and Economics,
Department of Applied Biotechnology and Food Science,
H-1111 Budapest, Műegyetem rkp. 3, Hungary

Original scientific paper
Received: May 25, 2009
Accepted: March 26, 2010

Batch fermentation experiments were performed to evaluate the potentials of different fractions of wheat as alternative carbon and nitrogen source for an economical production of lactic acid by a homofermentative mesophilic bacterium (*Lactobacillus* sp. MKT-878 NCAIM B02375). Hydrolysing the starch content of wheat results in well consumable glucose solution, and simultaneously by 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. By means of an optimization process we found that the gluten fraction can substitute the major part of the added yeast extract as nitrogen source, and on the basis of a statistical experimental design we created an optimized medium with 8 g L^{-1} yeast extract and 16 g L^{-1} gluten supplementation, resulting in 3.54 g $L^{-1}h^{-1}$ productivity which can be considered as an industrially acceptable process output.

Key words:
Gluten, hydrolysis, lactic acid, wheat, yeast extract

Introduction

Research on the fermentative lactic acid production has a very long history. Although lactic acid has very wide application possibilities, its usage was limited, because the production costs of lactic acid derivatives were higher than the similar products of petrolindustry origin. In the last decade, the price of mineral oil has increased dramatically, which has enabled the decrease of cost differences between petrolindustrial products and biotechnology products, and thus the application of lactic acid can be extended to low cost – high value derivatives.[1]

Lactic acid has both food- and chemical industrial applications, and it can be easily produced by microorganisms from different raw materials; it also serves as an appropriate starting-point for several compounds.[1] In the focus of these developments, there are several cost-decreasing solutions, since the low-value/high-volume products can only be economically feasible if the production costs are extremly minimized. 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 demand of lactic acid bacteria without no or with a minimal amount of supplementation.

In 2008, Hungary produced 4.5 million t of wheat with a 2.8 million t internal consumption, and although an annual average of 2.5–3 million t of wheat has food quality,[2] in the last few years it has shown up a claim to use surplus grain capacity in white-industrial technologies.[3] Accordingly, a concept called "Hungarian bio-refinery" was developed with a target to produce lactic acid as the main product from wheat utilizing the whole wheat grain. While the starch content of the wheat after hydrolysis resulting in glucose serves as a carbon source for lactic acid bacteria, the other components (gluten, bran etc.) are also precious by-products of the technology. They can be used either in the production of biogas or for direct commercialization. The main product – lactic acid – can then be converted via simple synthetic steps to other chemicals such as poly-lactate (PLA), butyl-lactate, ethyl-lactate, propylene-glycol etc.

It is known that lactic acid bacteria can utilize the hydrolysate of wheat starch,[4–7] but these bacteria require a high level of nutrient supplementation including nitrogen source, amino acids, vitamins and microelements.[8–12] To cover these needs, yeast extract is generally added to the media as the best nutrient source,[13] however the use of yeast extract as only nitrogen and additional nutrient source makes the technology extremely costly. Corn steep liquor, yeast autolysate, peptone, tryptone etc. may also come into question as alternative organic nitrogen sources, and after a proteolytic digestion the

*E-mail: bsevella@mail.bme.hu, Phone/Fax: 00 36 1 463 26 93

protein fraction of wheat (gluten) can also be utilized for fermentation purposes. The proteolysis of gluten can be carried out either separately or simultaneously with the starch hydrolysis,[14] 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,[15–16] helping the microbes to utilize gluten fraction and at the same time ameliorating the rheological properties of the medium. Accordingly, hydrolyzing and using the protein fraction of wheat can mostly substitute yeast extract, and as commercial ß-glucoamylase enzyme products applied in the brewing industry have protease activity as well, we do not need to calculate on extra costs of protease enzymes.

Utilization of starch content of wheat as carbon source and the protein content as nitrogen source is reported earlier,[4–7] and it may prove a great advantage toward to a more economical lactic acid production process to replace expensive nutrient supplementations. In this report, we are presenting our efforts on media optimization considering low cost supplementations.

Materials and methods

Microorganism

Lactobacillus sp. MKT-878 NCAIM B02375, 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.

Culture conditions

Precultures for laboratory fermentation experiments 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 transfer loop. These experiments were carried out in 250-mL shaking 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) fermentors were employed for laboratory fermentations.

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 calculated on the starting sugar concentration). 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 w = 25 % H_2SO_4 and w = 25 % NH_4OH.

The flasks, the neutralizing $CaCO_3$ 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).

Media and hydrolysis

Raw materials

The base of media was wheat flour type 550 (all-purpose flour, with w = 0.55 % ash fraction) with a protein content of 11 % dry mass and a wet gluten fraction of 27 %. The starch fraction (65 % dry mass) of wheat flour was degradable to glucose before fermentation in two steps, by liquefaction of starch and by saccharification of oligosaccharides (separate hydrolysis and fermentation).

Three types of media were prepared.

Basic wheat flour medium I. (without gluten): after adding 166 mL tap water to 200 g wheat flour, 1 hour kneading and addition of 208 mL tap water and 16.4 µL Shearzyme® 500 L enzyme (Novo Nordisk, Denmark) was carried out for the protein 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 1000 mL. After preparing gluten-free medium starch hydrolysis steps were performed.

Basic wheat flour medium II. (with gluten): 200 g wheat flour was suspended in tap water to a final volume of 1000 mL, then starch hydrolysis steps were performed.

Optimized wheat flour medium (with gluten and yeast extract): after gluten separation of basic wheat flour medium I. vital gluten (Victory 2001 Ltd., Hungary) and yeast extract (Reanal Budapest, Hungary) were added in an appropriate ratio, and the liquefaction and saccharification process was carried out.

The additional supplements, i.e. yeast extract, 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. 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.

Hydrolysis

For hydrolysis steps we followed instructions of Novo Nordisk product sheets.

In 1 L of wheat flour based medium the liquefaction of starch was carried out for 40 min by

56 µL Termamyl® SC (α-amylase by Novo Nordisk), at 85 °C and pH 5.5. For the saccharification (separately and prior to fermentation) 160 µL SAN® Super 240 L (glucoamylase and protease by Novo Nordisk), for 46 h, at 55 °C and pH 5.5 was used.

Since the SAN® Super 240 L product of Novo Nordisk contains proteases as well, the hydrolysis of gluten was done in line with saccharification (at 55 °C and pH 5.5).

Hydrolysis was performed in a 2-liter B. Braun Biostat® M fermentor (500 rpm).

Analyses

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

Cell growth was measured as optical density (Pharmacia LKB-Ultrospec Plus Spectrophotometer) at a wavelength of λ = 600 nm. In the case of shaking flasks 0.2 mol L^{-1} HCl was added to the samples (tenfold dilution) to dissolve the residual $CaCO_3$ but it resulted precipitation of wheat proteins which disturbed the optical density measurements. Accordingly, we did not measure the optical density of shaking flask samples.

The lactic acid production results of medium optimization were subjected to statistical analysis by Statistica v8 software.

Results and discussion

Fermentation on wheat starch hydrolysate without gluten and supplementations

In the first experiments, the glucose fraction of wheat starch hydrolysate gave the carbon source for the bacteria and it proved to be sufficient for the fermentation. The nitrogen source was only the water-soluble protein fraction of wheat flour type 550 which is w ~ 2.5 % of dry mass. There was no further supplementation, the necessary minerals were assumed to be stemmed from wheat and tap water.

The conversion was slow (Fig. 1) and incomplete; after a week, cultivation the broth still contained 60 g L^{-1} residual glucose. This indicated the need of more supplementation for the growth requirements of the lactic acid bacteria.

Obviously, applying this poor additive content starch hydrolysate as only nutrient source that resulted in as low as Y = 64 % lactate yield and P = 0.22 g L^{-1} h^{-1} productivity proved to be unfeasible to reach industrially acceptable process outputs.

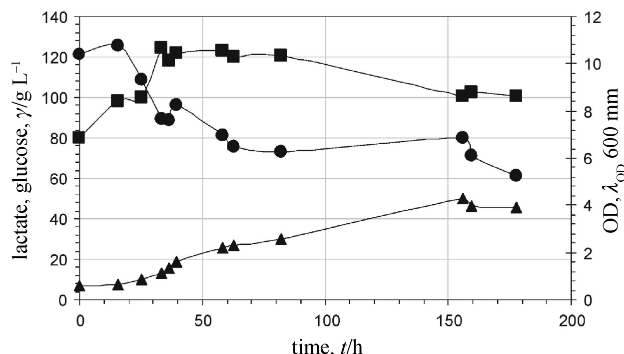


Fig. 1 – *Lactic acid fermentation on wheat starch hydrolysate without gluten and other supplements: (●) glucose; (▲) lactic acid; (■) optical density*

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 potential nitrogen sources: yeast extract, corn steep liquor and yeast autolysate.

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

Corn steep liquor, the by-product of the wet corn-starch producing technology, which contains several vitamins and diverse nitrogen sources is much cheaper, since it is an agro-industrial by-product, thus it has a positive effect on the lactic acid 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.

As shown by the results in Table 1, 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 unavailable in Hungary. The required total nitrogen amount is 2 g L^{-1} in the case of yeast autolysate, i.e. 96 g L^{-1} (wet mass) 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 Y = 93 %, productivity of P = 1.48 g $L^{-1}h^{-1}$). Nevertheless, the enormous amount (96 g L^{-1}) of the required yeast and

Table 1 – *Effect of nitrogen-supplementation quality and quantity on lactic acid productivity*

Nitrogen--supplementation	Amount of supplementation (γ/g L^{-1})	Total nitrogen--content (γ/g L^{-1})	Productivity (48 h) (P/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

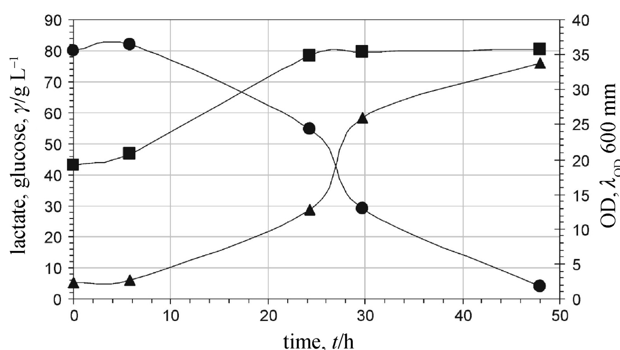


Fig. 2 – *Lactic acid fermentation on optimized wheat starch hydrolysate without gluten: (●) glucose; (▲) lactic acid; (■) optical density*

the complicated previously necessary inactivation of yeast cells and their alcohol producing enzyme system, makes this supplementation inconvenient for a feasible technology.

Effect of yeast extract as supplementation for lactic acid fermentation is also confirmed by Nancib *et al.*,[10] who compared its effect with other nitrogen-containing supplements (among others with corn steep liquor) and it clearly showed the greatest enhancing effect on lactic acid production.

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 the use of the whole protein mass fraction of wheat (w = 11 %). The gluten is the insoluble protein mixture of wheat, thus it is not directly usable as medium component. With 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 becomes usable as fermentation medium.

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

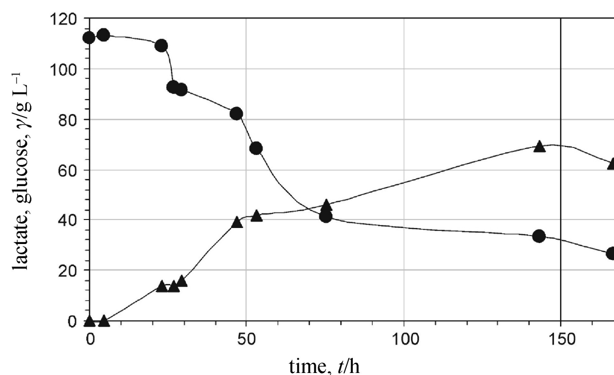


Fig. 3 – *Lactic acid fermentation on wheat flour hydrolysate with gluten: (●) glucose; (▲) lactic acid*

Optimization of wheat flour hydrolysate-based medium (with gluten)

To find the abovementioned minimum amount of yeast extract (YE) for glucose bioconversion (as the source of nitrogen or other necessary components), the medium was supplemented by YE in different concentrations (Table 2). Nancib *et al.*[10]

Table 2 – *Effect of yeast extract supplementation on lactic acid productivity*

Nitrogen--supplementation	Amount of supplementation (γ/g L^{-1})	Total nitrogen--content (γ/g L^{-1})	Productivity[a] (48 h) (P/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

[a]Productivity results were calculated at zero residual sugar concentration

showed that the combination of yeast extract with a less expensive nitrogen source could be economically used to reduce yeast extract supplementation without significant decrease in the lactic acid production rate.

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.

Table 3 – *Effect of yeast extract and gluten supplementation on lactic acid productivity*

Ingredient		Productivity (24 h) (P/g L^{-1} h^{-1})	Average
gluten	yeast extract		
concentration used (γ/g L^{-1})			
0	1	0.99	0.87
0	1	0.74	
0	5	1.31	1.23
0	5	1.16	
0	10	1.71	1.58
0	10	1.45	
10.2	1	1.72	1.72
10.2	1	1.72	
10.2	5	2.52	2.52
10.2	5	2.51	
10.2	10	2.66	2.66
10.2	10	2.65	
20.4	1	1.60	1.57
20.4	1	1.54	
20.4	5	3.01	2.80
20.4	5	2.59	
20.4	10	3.16	3.10
20.4	10	3.03	

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

In the investigation of Hofvendhal and Hahn-Hagerdal[7] in the case of two strains of

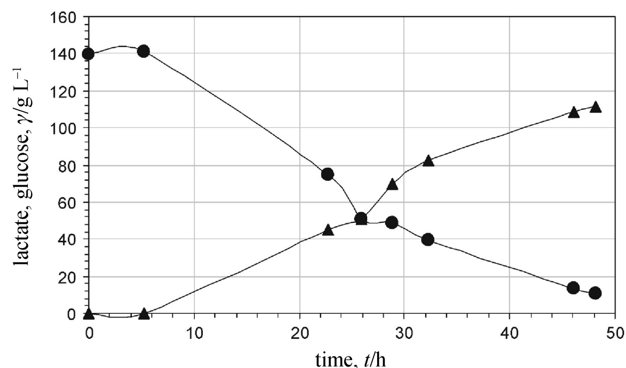


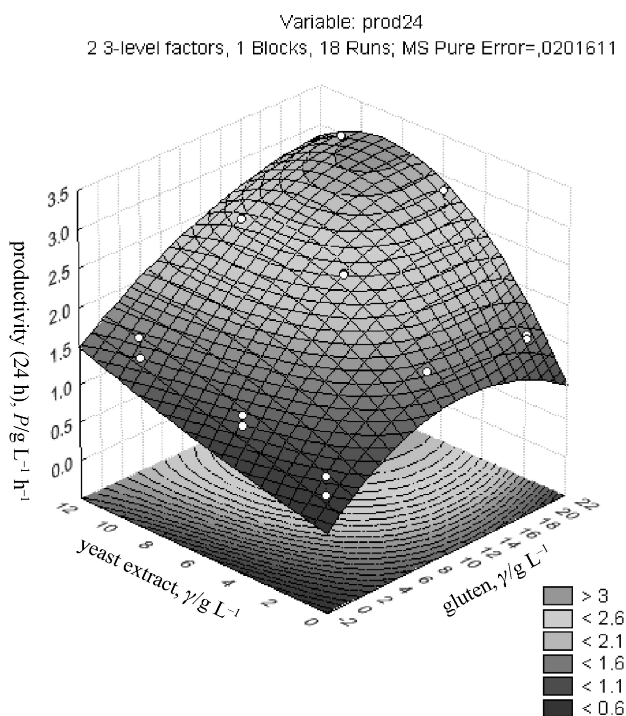
Fig. 4 – *Lactic acid fermentation on optimized wheat flour hydrolysate with gluten: (●) glucose; (▲) lactic acid*

lactococci and two strains of lactobacilli, the addition of a minimal amount of yeast extract (5 g L^{-1}) resulted in 0.3–2 g L^{-1} h^{-1} increase in maximal productivity. However, the total productivity (calculated by the total duration of fermentation) did not reach the 1 g L^{-1} h^{-1} value in any case.

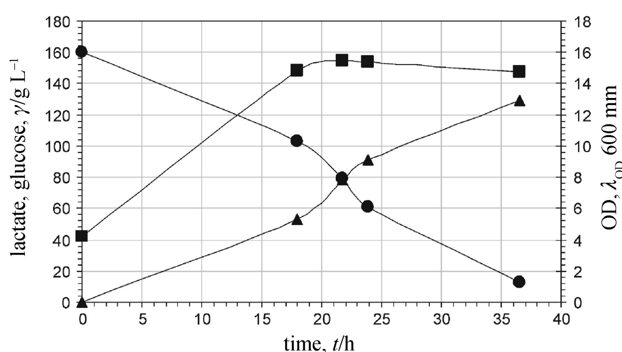
The results above show that yeast extract has a role as microelement-supplementation and gluten can cover the nitrogen demand of lactobacilli in an appropriate amount. To increase the lactic acid production rate by minimal addition of yeast extract and gluten, we had to find the optimal ratio of these supplements. An experimental design was generated with 9 factor levels (2^3) which were done in duplicate for a total number of 18 experiments. Table 3 contains the settings of the experimental design and the productivity results of fermentations. Productivity results of 48-hour fermentation did not show relevant differences to evaluate the effects of the factors (gluten and yeast extract), hence we evaluated the results after 24 h.

In the experimental design generated by the Statistica program, we fitted a second-order polynomial model to our data with two-way interactions, and the model was adequate. According to ANOVA analysis, both the yeast extract and the gluten had significant effects on lactic acid productivity ($p < 0.05$). The response surface (Fig. 5) shows the appropriate ratio of supplements where lactic acid productivity reaches its maximum. These optimal amounts of supplements are 8 g L^{-1} and 16 g L^{-1} for yeast extract and gluten respectively.

The lab scale fermentation experiment with this optimized yeast extract-gluten ratio gave a considerably high outcome in lactic acid production with a yield of Y = 88 % and a productivity of P = 3.54 g L^{-1} h^{-1} (Fig. 6).



F i g . 5 – *Response surface plots representing the 24 hours productivity results (g L^{-1} h^{-1}) from experimental design*



F i g . 6 – *Lactic acid fermentation on optimized wheat flour hydrolysate with gluten and yeast extract: (●) glucose; (▲) lactic acid; (■) optical density*

Conclusion

Nowadays lactic acid production from renewable resources is feasible via fermentation, and several microorganisms are able to produce this fermentation product which can be the starting-point for a number of compounds such as poly-lactate, propylene-glycol, ethyl-lactate etc. The object of this work was a new approach in using wheat flour for lactic acid fermentation purposes: however, utilizing wheat and its carbon and nitrogen contents for lactic acid fermentation is not a novelty, optimization of this wheat-based media has never been described before.

The hydrolysis of wheat starch and proteins by commercial enzyme products obviously covers the carbon and nitrogen demands of bacteria. The use of wheat gluten as a nitrogen source significantly reduces the need for yeast extract or other nitrogen-containing supplementation, which otherwise would make 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. Nevertheless, yeast extract cannot be totally substituted by wheat gluten because of its microelement-supplementation role. Based on the shake flask experiments run according to a statistical experimental design in order to find the appropriate ratio of gluten and yeast extract, we succeeded in optimizing the wheat flour-based medium that resulted in a yield of 88 % and a productivity of 3.54 g L^{-1} h^{-1}. This may be a good starting point for further technology optimization efforts.

ACKNOWLEDGMENT

This work was supported by HUNEST Biorefinery Ltd. We also wish to thank Palus Ltd. for submitting Novozymes enzyme products for hydrolysis processes.

List of symbols

c – concentration, mmol L^{-1}
P – volumetric productivity, g $L^{-1}h^{-1}$
Q – volumetric flow rate, mL min^{-1}
t – time, h
w – mass fraction, %
Y – yield, %
γ – mass concentration, g L^{-1}
λ – wavelength, nm

References

1. *Rojan, P. J., Nampoothiri, K. M., Pandey, A.*, Appl. Microbiol. Biotechnol. **74** (2007) 524.
2. *Kovács, T.*, Agroinform **2** (2009), Agroinform web portal: http://www.agroinform.com/aktualis/6975/A_magyar_búza_piaci_kilátásai.html.
3. *Hetényi, K., Németh, Á., Sevella, B.*, Hungarian Journal of Chemistry **3** (2008) 102.
4. *Akerberg, C., Hofvendahl, K., Zacchi, G., Hahn-Hagerdal, B.*, Appl. Microbiol. Biotechnol. **49** (1998) 682.
5. *Akerberg, C., Zacchi, G.*, Bioresour. Technol. **75** (2000) 119.
6. *Hofvendahl, K., Akerberg, C., Zacchi, G., Hahn-Hagerdal, B.,* Appl. Microbial. Biotechnol. **52** (1999) 163.

7. *Hofvendahl, K., Hahn-Hagerdal, B*., Enzyme Microb. Technol. **20** (1997) 301.
8. *Hofvendahl, K., Hahn-Hagerdal, B*., Enzyme Microb. Technol. **26** (2000) 87.
9. *Krishnan, S., Prapulla, S. G., Rajalakshmi, D., Misra, M. C., Karanth, N. G*., Bioprocess. Eng. **19** (1998) 61.
10. *Nancib, N., Nancib, A., Boudjelal, A., Benslimane, C., Blanchard, F., Boudrant, J*., Bioresour. Technol. **78** (2001) 149.
11. *Oh, H., Wee, Y.-J., Yun, J.-S., Han, S. H., Jung, S., Ryu, H.-W*., Bioresour. Technol. **96** (2005) 1492.
12. *Olmos-Dichara, A., Ampe, F., Uribelarrea, J.-L., Pareilleux, A., Goma, G*., Biotechnol. Lett. **19** (1997) 709.
13. *Nancib, A., Nancib, N., Meziane-Cherif, D., Boubendir, A., Fick, M., Boudrant, J*., Bioresour. Technol. **96** (2005) 63.
14. *Rojan, P. J., Nampoothiri, K. M., Pandey, A*., Biotechnol. Lett. **28** (2006) 1823.
15. *Kong, X., Zhou, H., Qian, H*., Food Chem. **101** (2007) 615.
16. *Kong, X., Zhou, H., Qian, H*., Food Chem. **102** (2007) 759.