Electronic Journal of Polish Agricultural Universities is the very first Polish scientific journal published exclusively on the Internet, founded on January 1, 1998 by the following agricultural universities and higher schools of agriculture: University of Technology and Agriculture of Bydgoszcz, Agricultural University of Cracow, Agricultural University of Lublin, Agricultural University of Poznan, Higher School of Agriculture and Teacher Training Siedlce, Agricultural University of Szczecin, and Agricultural University of Wroclaw.

ELECTRONIC
JOURNAL
OF POLISH
AGRICULTURAL
UNIVERSITIES

2000
Volume 3
Issue 2
Series
FOOD SCIENCE AND TECHNOLOGY

NOWAK J. 2000. ETHANOL YIELD AND PRODUCTIVITY OF *ZYMOMONAS MOBILIS* IN VARIOUS FERMENTATION METHODS **Electronic Journal of Polish Agricultural Universities**, Food Science and Technology, Volume 3, Issue 2.
Available Online http://www.ejpau.media.pl

ETHANOL YIELD AND PRODUCTIVITY OF *ZYMOMONAS MOBILIS* IN VARIOUS FERMENTATION METHODS

Jacek Nowak
Institute of Food Technology of Plant Origin, Agricultural University of Poznań, Poland

ABSTRACT

Ethanol producing bacteria *Zymomonas mobilis* (strain 3881 and 3883) were used in batch and continuous fermentation as free cells as well as immobilized in alginate beads. Continuous fermentation helped to increase the productivity of fermentors significantly and continuous fermentation with immobilized in alginate cells gave as high productivities as 49,5 g/dm^3*h.

Key words: *Zymomonas mobilis*, batch and continuous ethanol fermentation, immobilization

INTRODUCTION

From other microorganisms than *Saccharomyces cerevisiae* tested as a potential ethanol producers, *Zymomonas mobilis* is probably the most suitable organism. It converts glucose almost stoichiometrically to ethanol and CO_2, grows more rapidly than the yeast and demonstrates highest productivity during continuous fermentation (Rogers *et al*. 1986, Buchholz and Eveleigh 1990, Qureshi and Manderson 1995). The bacteria present significantly higher specific rates of sugar uptake and ethanol production compared to those found for yeasts (Rogers *et al*. 1986, Lawford 1988, Nowak 1999). *Zymomonas* cultures grow anaerobically and unlike yeasts do not require the controlled addition of oxygen to maintain viability at high cell concentrations (Rogers *et al*. 1986, Karsch *et al*. 1983). Ethanol tolerance of some strains of *Zymomonas* is comparable if not higher than strains of *Saccharomyces cerevisiae* (Rios *et al.* 1991, Busche *et al.* 1992). *Zymomonas mobilis* produce less by-products, especially fusels (Gałaj *et al.* 1994, Nowak *et al.*1997). The genetic manipulation of *Zymomonas* is simpler than for yeasts which give the opportunity to widen the spectrum of raw materials for ethanol production to lignocellulosic materials and direct digestion of starch (Zhang *et al.* 1995, Dumsday *et al.* 1997, Nowak 1998). Continuous techniques of fermentation are especially suitable for this microorganisms and the productivities of bacteria are much more higher then yeasts (Busche *et al.* 1992, Nowak 1999).
In this work the fermentation capabilities of two *Zymomonas mobilis* strains from Czech Culture Collection were tested using both batch and continuous methods as well as immobilization of cells in alginate. Advantages of continuous technique with immobilized bacteria cells were demonstrated.

MATERIALS AND METHODS

- Bacterial culture *Zymomonas mobilis* 3881 and 3883 from Czech Culture Collection were used.
- *Z. mobilis* was cultured on glucose medium (glucose 80 g/dm^3, yeast extract 10 g/dm^3, KH_2PO_4 1 g/dm^3, $(NH_4)_2SO_4$ 1 g/dm^3, and $MgSO_4 \times 7H_2O$ 0.5 g/dm^3).
- Glucose medium (glucose 80 to 250 g/dm^3, yeast extract 10 g/dm^3, KH_2PO_4 1 g/dm^3, $(NH_4)_2SO_4$ 1 g/dm^3, and $MgSO_4 \times 7H_2O$ 0.5 g/dm^3) was used in the experiments.
- Batch fermentation were done in 0.5 dm^3 Erlenmeyer flask containing 0.2 dm^3 medium and 0.02 dm^3 of inoculum (80 g/dm^3 glucose medium after 24 hours fermentation at 30^{o}C).
- When immobilized in calcium alginate cells were tested, 40 g of alginate beads (number of bacterium cells in 40 g of alginic beads just after immobilization *Zymomonas mobilis* 3881 = 1.02×10^{11}, *Z.m*. 3883=9.18×10^{10} , the amount of glucose in the medium on the start 147g/l for *Z.m*. 3881 and 141 g/l for Z.m. 3883) were used and flasks were incubated in water bath shaker (150 rpm) at 30^{o}C.
- For continuous fermentation BioFlo C 30 fermentor (New Brunswick) was used with 1 dm^3 reactor (0.38 dm^3 working volume) and 0.038 dm^3 of inoculum (80 g/dm^3 glucose medium, 30^{o}C) was added and after 24 hours culturing the continuous process was started.
- For immobilization sodium alginate solution (30 g/dm^3) was mixed with bacterial cells resuspended in sterile water, added dropwise to 0.1 mol/dm^3 $CaCl_2$ and allow to solidify for 1 h. 180 g gel beads were used in the bioreactor. Bacterial cells were centrifuged (3000 rpm, K70, 10 min.) after 24 hours fermentation on 80 g/dm^3 glucose medium at 30^{o}C. In 180 g of alginate 3.3-3.5 g d.m. of bacterial cells were immobilized.

- All the fermentations were carried out at 30°C for 24 hours (100 g/dm^3 glucose medium, batch fermentation to 96 hours (250 g/dm^3 glucose medium, batch fermentation) or 15 to 30 days (continuous fermentation).
- Sugars were estimated spectrophotometrically using 3.5-dinitrosalicylic acid and glucose as a standard (Miller 1959).
- Ethanol was measured by distillation.
- Cell biomass was estimated by drying in 60°C with ethanol and then in 105°C to a constant weight or counted directly in a homocytometer.
- Ethanol yield was counted for used sugars and the amount of sugars in the medium after sterilization and after fermentation was estimated for each sample. The theoretical ethanol production was calculated for the sugars used and taken as 100%. The productivity was expressed as the amount of ethanol (g) formed in bioreactor within one hour calculated for one dm^3 of working volume of fermentor.

RESULTS AND DISCUSSION

The most popular medium used for testing *Z. mobilis* ethanol production was glucose medium with yeast extract, ammonium sulfate and magnesium sulfate (Karsch *et al*. 1983, Struch *et al*. 1991, Agrawal and Veeramallu 1990, Oaxaca and Jones 1991, Falcao de Morais *et al*. 1993, Nowak and Roszyk 1997, Rios *et al*. 1991).

Ethanol yield of 2 bacterial strains on glucose medium containing from 100 to 250 g glucose in 1 dm^3 using batch fermentation were compared in table 1. The ethanol yield was ranging from 91.8 to 96% of theoretical and glucose utilization was high for both strains even in medium with high sugar concentration. Glucose from 250 g/dm^3 medium was utilzed only in the level of 91.2% when *Z. mobilis* 3881 strain was used. This strain was less tolerant to the high glucose content in the medium than the other one. The physiological basis of the exceptionally high sugar tolerance of *Zymomonas* was investigated by Struch *et al*. (1991). *Z. mobilis* has a facilitated diffusion system which enables a rapid equilibration between internal and external glucose concentration. Close to the theoretical ethanol yield for bacteria for media with high glucose concentration is likely to be connected with both less metabolic energy used for growth of population and less maintenance energy used by bacteria biomass in that situation than yeasts (Nowak 1999).

Table 1. Ethanol yield and glucose utilization by two *Z. mobilis* strains in batch fermentations (30°C, fermentation time 48h and 72h for medium 250 g glucose/l).

Strain	Glucose (100g/dm^3)		Glucose (150g/ dm^3)		Glucose (200g/ dm^3)		Glucose (250g/ dm^3)	
	Ethanol yield (% of theoretical)	Glucose used (%)	Ethanol yield (% of theoretical)	Glucose used (%)	Ethanol yield (% of theoretical)	Glucose used (%)	Ethanol yield (% of theoretical)	Glucose used (%)
3881	93.8^a	98.2	91.8^a	98.5	93.6^a	97.9^a	95.9	91.2^a
3883	94.3^b	98.5	93.4^b	98.6	94.2^b	99.0^b	96.0	98.2^b

Means within columns with different letters differ significantly (α =0.05)

Immobilization seems to be a very promising technique in a number of fermentation processes. Also employed to ethanol production in batch fermentation let to use the same cells for a fermentation few times in repeated fermentations. Yields were however lower for both *Zymomonas* strains (tab.2). As the pH falls down up to 3.3-4.2 in immobilized batch fermrntation

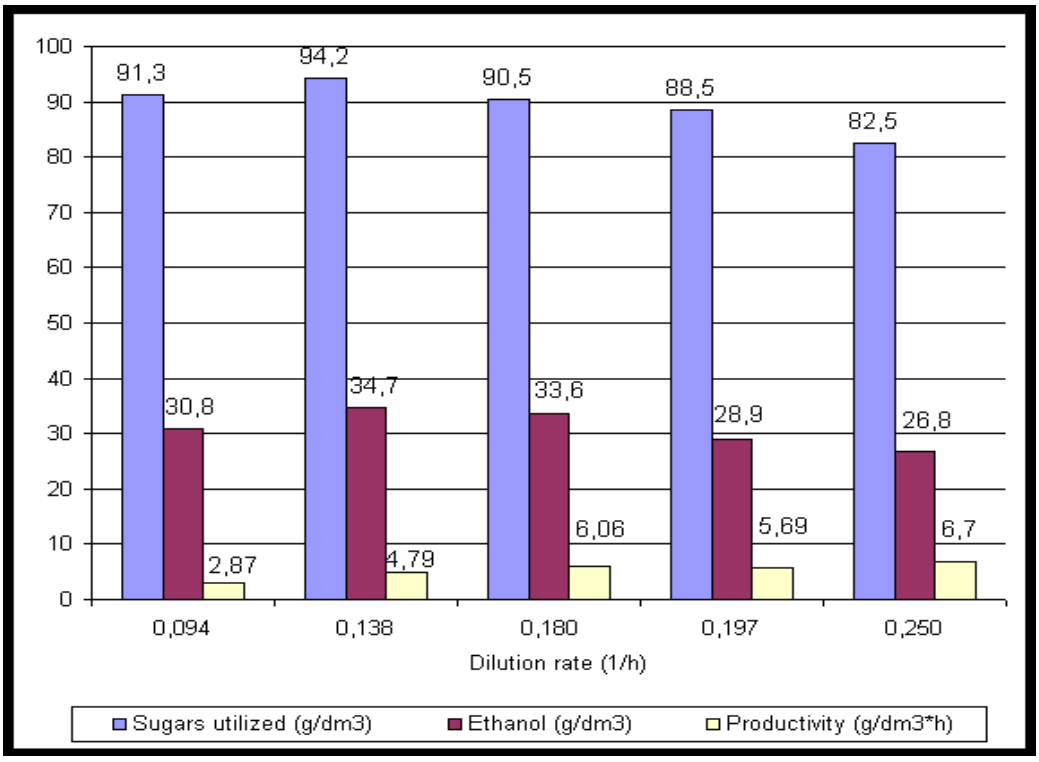
of glucose medium (normally pH is 4.6-5.0 in batch bacteria fermentation) regulation of pH for 5.2 was used. Still while the sugar utilization from 150 g glucose/dm^3 medium was high, the yield was much less than 90% of theoretical. These facts might be partly connected with the high level of produced during fermentation acids (decrease of pH) to produce which bacteria use some of metabolic energy from glucose. It is also the evidence that in the presence of acetic acid *Z. mobilis* produce ethanol with 8% less yield (Lawford and Rousseau 1992,1993).

Table 2. Ethanol yield and sugar utilization during batch fermentation using immobilized *Z. mobilis* 3881 and 3883 (pH controlled 5.2, temp. 30°C, 150 rpm)

Run (change of medium every 48 h)	Ethanol (% w/v)		Ethanol yield (% of theoretical)		Glucose used (%)	
	Z.m. 3881	Z.m. 3883	Z.m. 3881	Z.m. 3883	Z.m. 3881	Z.m. 3883
1	5.26 ± 0.04	6.06 ± 0.04	72.46 ± 0.57	85.87 ± 0.58	96.67 ± 0.02	97.86 ± 0.02
2	6.31 ± 0.07	6.24 ± 0.07	86.07 ± 0.99	88.46 ± 1.00	97.65 ± 0.02	97.87 ± 0.03
3	6.53 ± 0.04	6.20 ± 0.22	89.04 ± 0.52	88.32 ± 1.13	97.69 ± 0.06	97.61 ± 0.02

Continuous fermentation of glucose medium (100 g/dm^3) was tested on 0.38 dm^3 working capacity bioreactor with gentle stirring (50 rpm). Dilution rates from 0.1 to 0.25 (h^{-1}) was used (Fig.1)

Figure 1. The influence of dilution rate on sugar utilization and ethanol production by *Z. mobilis* 3883 on glucose medium (100 g/l) during continuous fermentation.



The yields of ethanol was lower than in batch fermentation again. Productivity reached only 6.7 g/dm^3*h for *Z. mobilis* 3883 which was still better then the productivity of another strain. Those relatively low values was connected with fact that the significant wash out of bacterial cells from bioreactor took place. It caused decrease of sugar utilization and ethanol production with growing dilution rate. The results were comparable to those cited in literature (Ishizaki *et al.* 1994).

Fig. 1. The influence od dilution rate on sugar utilization and ethanol production by *Z. mobilis* 3883 on glucose medium (100 g/l) during continuous fermentation.

The same bioreactor BioFlo C30 (New Brunswick) was used, filled with 180 g of alginic beads containing immobilized bacteria or yeasts. Gentle stirring (50 rpm) was employed which did not destroyed the structure of gel.

Using the immobilized system higher dilution rates could be applied which markedly increased the productivities of the used bioreactor. The glucose concentration in the medium was also increased to 130 g/dm^3. Dilution rates from 0.2 h^{-1} to more than 1.0 h^{-1} were tested. Different profile of reaction to the increase of dillution rate were obtained for the two tested strains. For strain *Z. mobilis* 3881 the productivity was increasing with the increase of amount of medium pumped to the bioreactor (dilution rate) with relatively stabile ethanol yield (Fig 2 and 3).

Figure 2. The influence of dilution rate on ethanol yield (% of theoretical) and bioreactor productivity during continuous fermentation of glucose medium by immobilized in alginate Z. mobilis 3881 cells

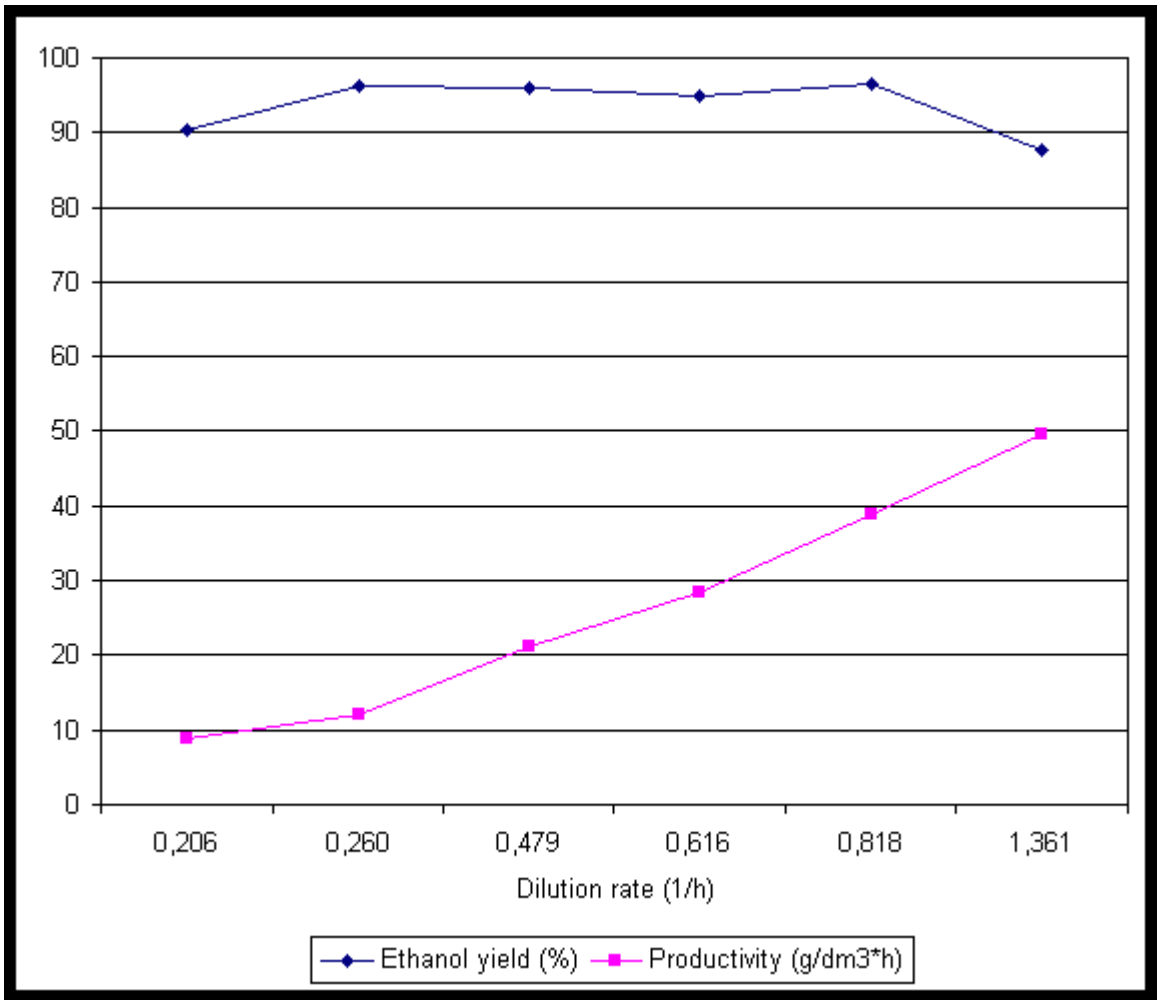


Figure 3. Sugars utilization, ethanol production and productivity during continuous fermentation 128 g/l glucose medium by immobilized *Z. mobilis* 3881.

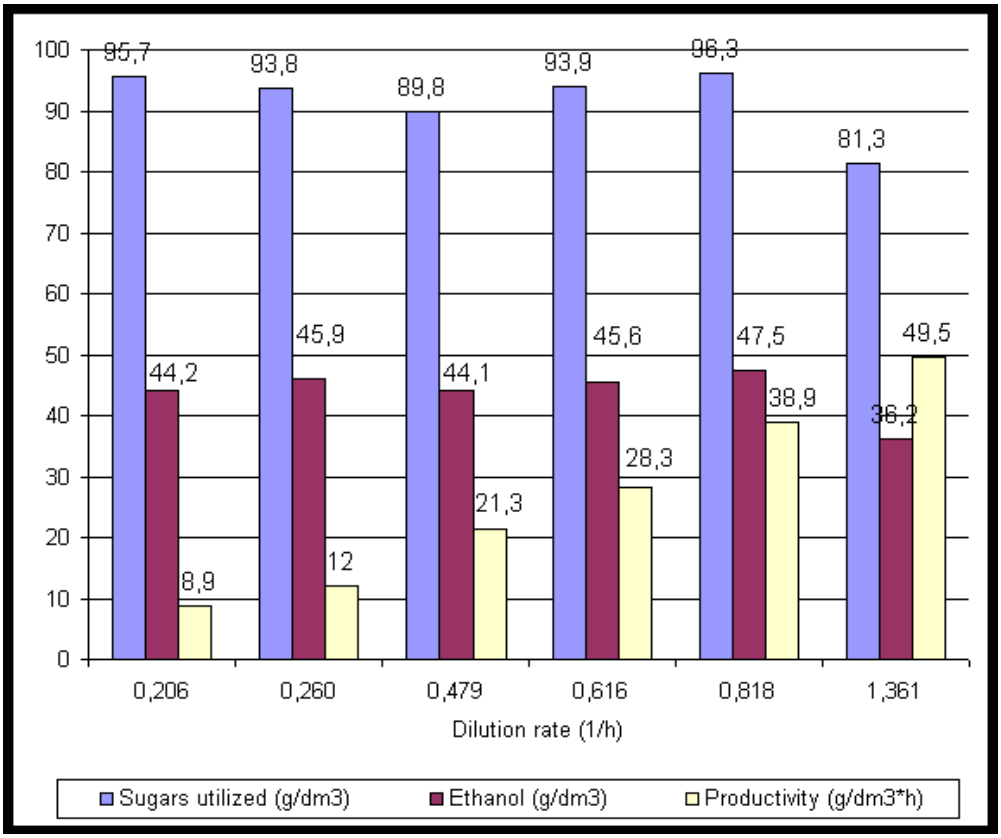


Figure 4. Sugars utilization, ethanol production and productivity during continuous fermentation 130 g/l glucose medium by immobilized *Z. mobilis* 3883

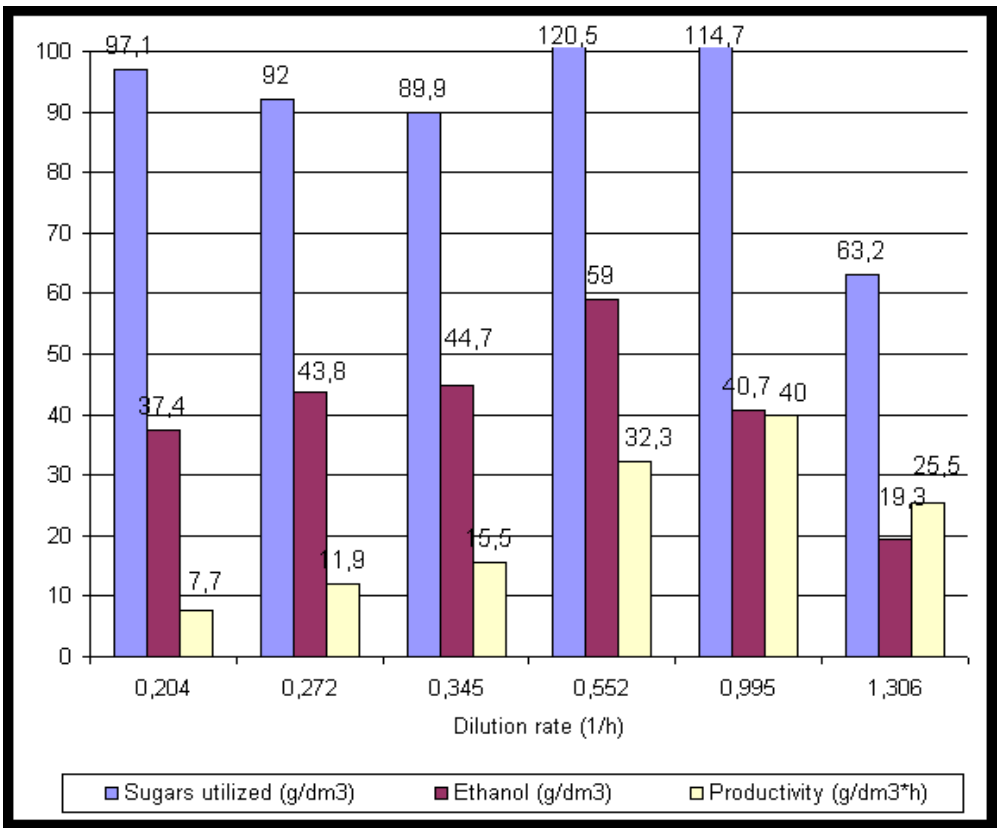
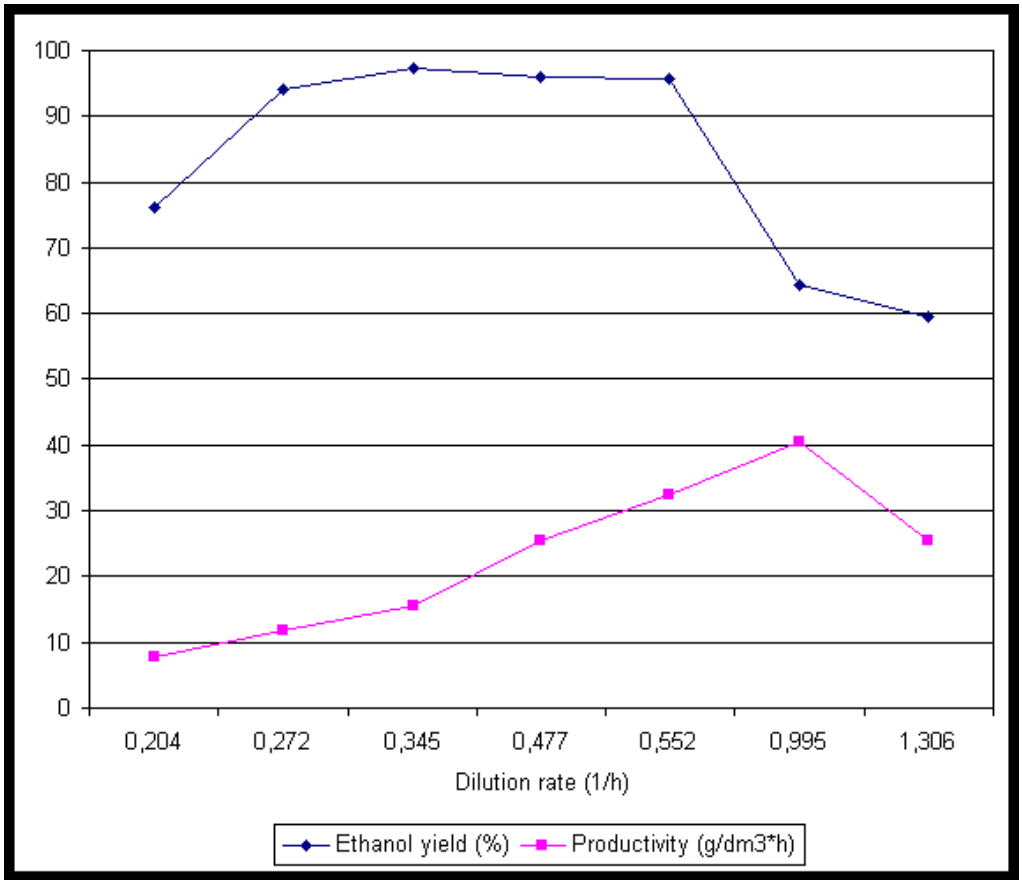


Figure 5. The influence of dilution rate on ethanol yield (% of theoretical) and bioreactor productivity during continuous fermentation of glucose medium by immobilized in alginate *Z. mobilis* 3883 cells



Strain *Z. mobilis* 3883 was less stabile and got its optimum in D=0.5 h^{-1} (productivity 32.3 g/dm^3*h, ethanol yield 96.6% of theoretical). The farther increase of dilution rate gave the higher productivity (40 g/dm^3*h) but the ethanol yield was dramatically lower (less than 70% of theoretical) (Fig. 4 and 5).

The productivities of ethanol during continuous fermentation with immobilized in alginate beads bacterial cells were high reaching 40-50 g/dm^3 of fermentor working volume in one hour. High productivity of this system decreases both the investment costs (low capacity of bioreactors) and operation costs in ethanol fermentation processes (Busche *et al.* 1992, Queresi and Manderson 1995).

The above advantages of bacterial continuous fermentation with immobilized cells, from the other hand, were demonstrated on rich glucose medium. There is evidence that the situation might be different when industrial media when rye mashes are used (almost 90% of ethanol is produced in Poland from rye). The yield of tested bacteria are the same as industrial yeasts in batch fermentation but difficulties with pumping the highly viscous medium makes continuous technique less useful (Nowak 1999). However there is to emphasise that bacteria are more suitable for ethanol production than yeasts, when more dynamic, continuous fermentation techniques are used, especially with immobilized cells.

As it seems that a serious challenge will concern bioethanol-producing industry in Central Europe when fuel bioethanol government programs will be opened, applying of more productive techniques and microorganisms must be taken into account. *Zymomonas mobilis* appears to have considerable potential for industrial alcohol production especially for high productivity fermentation systems.

REFERENCES

1. Agrawal R., Veeramallu U.: Ethanol fermentation by *Zymomonas mobilis* ATCC 10988 in repeated batch cultures. *J.Chem.Technol.Biotechnol.* 47, 1-6 (1990).
2. Buchholz S.F., Eveleigh D.F.: Genetic modification of *Zymomonas mobilis. Biotech. Adr.* 8, 547 – 566 (1990).
3. Busche R. M., Scott C. D., Davison B. H., Lynd L. R.: Ethanol, the ultimate feedstock. A technoeconomic evaluation of ethanol manufacture in fluidazed bed bioreactors operating with immobilized cells. *Appl. Biochem. Biotechnol.* 34/35, 395-415 (1992).
4. Dumsday G.J., Jones K., Stanley G.A., Pamment N.B.: Recombinant organisms for ethanol production from hemicellulosic hydrolyzates. *Australian Biotechnology* 5, 285-295 (1997).
5. Falcao de Morais J.O., Rios E.M.M., Calazans M., Lopes C.E.: *Zymomonas mobilis* research in the Pernambuco Federal University. *J. Biotech.* 31, 75 – 91 (1993).
6. Gałaj M., Pietrzykowska M., Włodarczyk Z.: Wstępne próby porównania fermentacji alkoholowej niektórych substratów bakteriami *Zymomonas mobilis* oraz drożdżami gorzelniczymi. *Przem. Ferm. i Owoc.-Warzyw.* 9, 13-15 (1994).
7. Ishizaki A., Tripetchkul S., Tonokawa M., Zhong-Ping S., Kazuyuki S.: pH mediated control methods for continuous ethanol fermentation using *Zymomonas mobilis*. *J.Ferment.Bioeng.* 77, 541-547 (1994).
8. Karsch T., Stahl U., Esser K.: Ethanol production by *Zymomonas* and *Saccharomyces*, advantages and disadvantages. *Eur.J.Appl.Microbiol.Biotechnol.* 18, 387-391 (1983).
9. Lawford H.G.: A new approach to improving the performance of *Zymomonas* in continuous ethanol fermentation. *Appl.Biochem.Biotechnol.* 17, 203-209 (1988).
10. Miller G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* 31, 426-428 (1959).
11. Nowak J.: Genetyczna modyfikacja drobnoustrojów do produkcji etanolu z hydrolizatów hemicelulozowych. *Biotechnologia* 3, 89-97 (1998).
12. Nowak J.: Bakterie *Zymomonas mobilis* – mikroorganizmy alternatywne dla drożdży gorzelniczych. *Roczniki Akademii Rolniczej w Poznaniu. Rozprawy Naukowe.* Zeszyt 300, Poznań 1999.
13. Nowak J., Czarnecki Z., Kamiński E.: The influence of fermentation technique and microorganism on spirits quality. Proceedings of the First Symposium "In Vino Analytica Scientia" pp. 431-434, Bordeaux, France 1997.
14. Nowak J., Roszyk H.: Co-immobilization of *Aspergillus niger* and *Zymomonas mobilis* for ethanol production from starch. *Pol.J.Food Nutr.Sci.* 6, 65-70 (1997).
15. Oaxaca V.A., Jones L.P.: Formation of ethanol and higher alcohols by immobilized *Zymiomonas mobils* in continuos culture. *Acta Biotechnol*. 11, 523-532 (1991).
16. Queresi N., Manderson G.J.: Bioconversion of renewable resources into ethanol: an economic evaluation of selected hydrolysis, fermentation, and membrane technologies. *Energy Sources* 17, 241-265 (1995).
17. Rios E. M., Salazans G. M. T., Antvnes R. V., Accidy L. G. A., Otamar J., Morais F.: Glucose fermentation by *Zymomonas mobilis* - effect of addition of ethanol and of cell free fermented must on the microbial growth and ethanol production. *Arg. Biol. Technol.* 34, 45-52 (1991).
18. Rogers P.L., Strzelecki A.T., Goodman A.E.: Commercial potential of *Zymomonas* process for ethanol production. *Found. For Biotech. Indust. Ferment. Res.* 4, 63 – 79 (1986).
19. Struch T., Neuss B., Bringer-Meyer S., Sahm H.: Osmotic adjustment of *Zymomonas mobilis* to concentrated glucose solutions. *Appl.Microbiol.Biotechnol.* 23, 201-211 (1991).
20. Zhang M., Eddy Ch., Deanda K., Finkelstein M., Picataggio S.: Metabolic engineering of pentose metabolism pathway in ethanologenic *Zymomonas mobilis. Science* 267, 240-243 (1995).

Submited:

Jacek Nowak
Institute of Food Technology of Plant Origin
Agricultural University of Poznań
31 Wojska Polskiego, 60-624 Poznań, Poland
e-mail: jacnow@owl.au.poznan.pl