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

2001
Volume 4
Issue 2
Series
FOOD SCIENCE AND TECHNOLOGY

NOWAK J. 2001. COMPARISON OF POLISH INDUSTRIAL DISTILLERY YEAST WITH ETHANOL PRODUCING BACTERIA *ZYMOMONAS MOBILIS* **Electronic Journal of Polish Agricultural Universities**, Food Science and Technology, Volume 4, Issue 2.
Available Online http://www.ejpau.media.pl

COMPARISON OF POLISH INDUSTRIAL DISTILLERY YEAST WITH ETHANOL PRODUCING BACTERIA *ZYMOMONAS MOBILIS*

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

ABSTRACT

Comparison between ethanol producing bacteria *Zymomonas mobilis* (strain CCM 3881 and CCM 3883) and commercial yeast (D_2, Bc-16a) was made using glucose medium. While the differences between bacteria and yeasts in low glucose medium (85.5 g/dcm^3) were not of statistical importance, ethanol yield and sugars utilization were higher for *Z. mobilis* on medium with high concentration of glucose (150-250 g/dcm^3). The bacteria growth was less inhibited by alcohol in the medium than yeasts. The bacteria biomass needed for 1 dcm^3 ethanol production was from 2 times (glucose medium 85.5 g/dcm^3) to 3-4 times (241 g glucose in 1 dcm^3 medium) less than yeasts.

Continuous fermentation helped to increase the productivity of bioreactors significantly and continuous fermentation with immobilized in calcium alginate beads gave as high productivities as 37 g/dcm^3 · h. Yeasts were proved to had much less productivities (half of that of bacteria when D=1.0 h^{-1}) and lower ethanol yield.

Key words: yeast, *Zymomonas mobilis*, ethanol yield, ethanol productivity, continuous fermentation

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 yeasts and demonstrates highest productivity during continuous fermentation [2, 20, 22]. Significantly higher specific rates of sugar uptake and ethanol production compared to those found for yeasts [11, 15, 22]. *Zymomonas* cultures grow anaerobically and unlike yeasts do not require the controlled addition of oxygen to maintain viability at high cell concentrations [10, 22]. Ethanol tolerance of some strains of *Zymomonas* is comparable and somethimes higher than strains of *Saccharomyces cerevisiae* [3, 21]. *Zymomonas mobilis* produces less by-products, especially fusels [7, 14]. The genetic manipulation of *Zymomonas* is simpler than for yeasts which give the opportunity to widen the spectrum of raw materials for ethanol pro duction to lignocellulosic materials and direct digestion of starch [5, 14, 24].

In this work the fermentation capabilities of two *Zymomonas mobilis* (CCM 3881 and CCM 3883) strains from Czech Culture Collection were compared with two stains of industrial Polish yeasts D_2 and Bc-16a. Both batch and continuous techniques were used as well as immobilization of cells in alginate in continuous process. Advantages of bacteria on glucose medium were demonstrated.

MATERIALS AND METHODS

- Dried Polish commercial yeast *Saccharomyces cerevisiae* Bc-16a and D_2 as well as bacterial culture *Zymomonas mobilis* CCM 3881 and CCM 3883 from Czech Culture Collection were used.

- Dried yeast were rehydrated 20 min in water (1:10) and acidified with H_2SO_4 (5 cm^3 in 1 dcm^3 water) for decontamination.

- *Z. mobilis* was cultured on glucose medium (glucose 80 g/dcm^3, yeast extract 10 g/dcm^3, KH_2PO_4 1 g/dcm^3, $(NH_4)_2SO_4$ 1 g/dcm^3, and $MgSO_4 \times 7\ H_2O$ 0.5 g/dcm^3).

- Glucose medium with different glucose level was used in the experiments.

- Glucose medium (glucose 80 to 250 g/dcm^3, yeast extract 10 g/ dcm^3, KH_2PO_4 1 g/dcm^3, $(NH_4)_2SO_4$ 1 g/dcm^3, and $MgSO_4 \times 7\ H_2O$ 0.5 g/dcm^3).

- Batch fermentation were done in 0.5 dcm^3 Erlenmeyer flask containing 0.2 dcm^3 medium and 10% of inoculum (80 g/dcm^3 glucose medium after 24 hours fermentation).

- For continuous fermentation BioFlo C 30 fermentor (New Brunswick) was used with 1 dcm^3 reactor (0.38 dcm^3 working volume) and 10 % of inoculum (80 g/dcm^3 glucose) was added and after 24 hours culturing the continuous process was started.

- For immobilization sodium alginate solution (30 g/dcm^3) was mixed with bacterial or yeast cells resuspended in sterile water, added dropwise to 0.1 mol/dcm^3 $CaCl_2$ and allow to solidify for 1 hour. Gel beads (180 g) were used in the bioreactor. Bacterial and yeast cells were centrifuged after 24 hours fermentation on 80 g/dcm^3 glucose medium (3000 min^{-1}, K70,

10 min). In 180 g of alginate beads 4.5-4.75 g d.m. of yeast cells and 3.3-3.5 g d.m. of bacterial cells were immobilized.

- All the fermentations were carried out in 30°C for 24-96 hours (batch fermentation) or 15 days (continuous fermentation).

- Sugars were estimated spectrophotometrically using 3,5-dinitrosalicylic acid and glucose as a standard [13]

- Ethanol was measured by density measurement after distillation.

- Cell biomass was estimated gravimetrically after drying at 60 ° C with ethanol and then at 105°C to a constant weight.

- Ethanol yield was calculated 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 in gram formed in bioreactor within one hour counting for one dcm^3 of working volume of fermentor.

RESULTS AND DISCUSSION

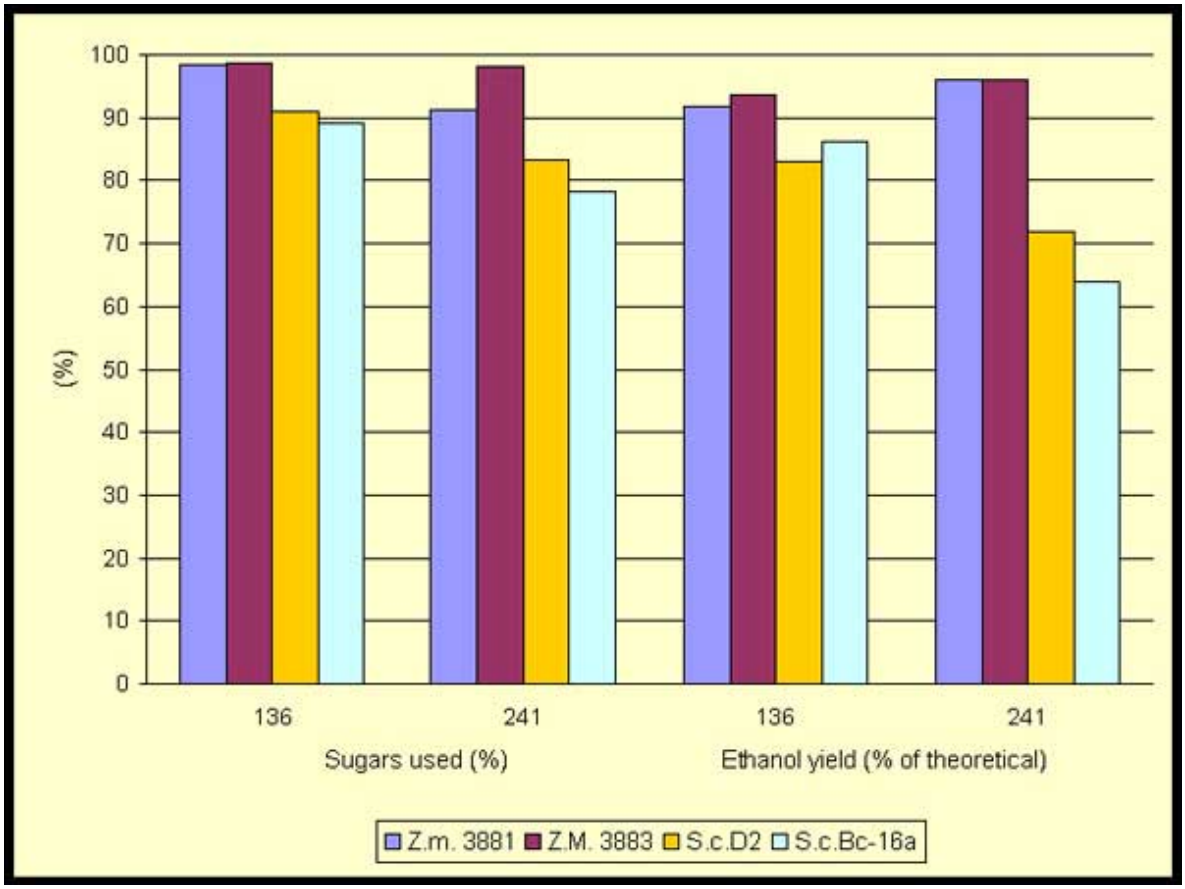
The most popular medium used for testing *Z. mobilis* ethanol production was glucose medium enriched in yeast extract, ammonium sulphate and magnesium sulphate [1, 6, 10, 17, 21, 23].

Ethanol yield of bacteria and yeast was very close when a glucose medium with lower level of glucose (85.5 g/dcm^3) was used (Tab. 1).Using medium with the amount of sugars normally present (136 g/dcm^3) in industrial mashes the yield for both types of microorganisms start to differentiate and the use of glucose in the amount close to 250 g in 1 dcm^3 medium proved that in high osmotic pressure circumstances bacteria producing ethanol with significantly higher yield (Fig. 1). The physiological basis of the exceptionally high sugar tolerance of *Zymomonas* was investigated by Struch et al. [23]. *Z. mobilis* has a facilitated diffusion system which enables a rapid equilibration between internal and external glucose concentration.

Table 1. Comparison of glucose (85.5 g/dcm^3) batch fermentation by *Z. mobilis* (3881, 3883) and *S. cerevisiae* (D_2, Bc-16a) (temp.30°C, 48 h, starting pH 5.2). Mean ± S.D.

Strain	Sugars used (g/ dcm^3)	Sugar utilization (%)	Ethanol (% w/v)	Ethanol yield (% of theoretical)	Biomass (g/dcm^3 fermentation broth)
3881	83.82 ± 0.04	98.01 ± 0.05	4.01 ± 0.15	93.60 ± 3.29	1.0585 ± 0.0870
3883	83.25 ± 0.19	97.35 ± 0.21	4.03 ± 0.08	94.08 ± 1.64	1.0560 ± 0.0840
D_2	83.96 ± 0.24	98.17 ± 0.27	4.03 ± 0.08	93.91 ± 2.62	2.6105 ± 0.0965
Bc-16a	83.55 ± 0.23	97.70 ± 0.26	4.03 ± 0.08	94.70 ± 1.65	2.0885 ± 0.2895

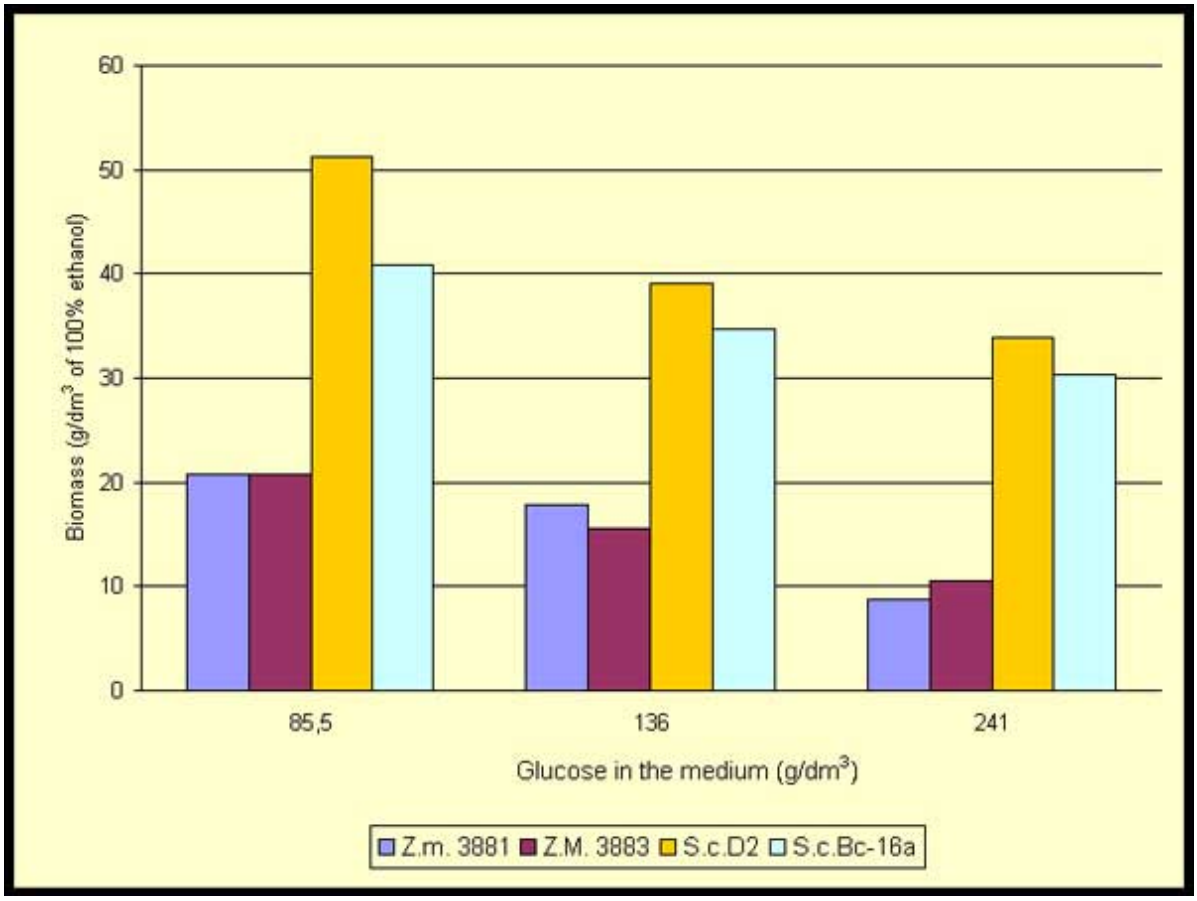
Fig. 1. Sugars utilization and ethanol yield (% of theoretical) on glucose media by bacteria *Z. mobilis* (3881 and 3883) and yeasts *S. cerevisiae* (D_2 and Bc-16a)



This is to stress that the amount of bacteria biomass produced during fermentation was less than yeast biomass.

The comparison of biomass needed to produce 1 dcm^3 of 100% ethanol proved, that with growing concentration of glucose in the medium the amount of biomass needed to form 1 dcm^3 ethanol decreased both for yeasts and bacteria. However to produce the same volume of pure ethanol we need significantly less bacterial biomass than yeast biomass (especially for the media with high glucose level). The bacteria biomass needed for 1 dcm^3 ethanol production was from 2 times (glucose medium 85.5 g/l) to 3-4 times (241 g glucose in 1 l medium) less than yeasts (Fig.2). 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.

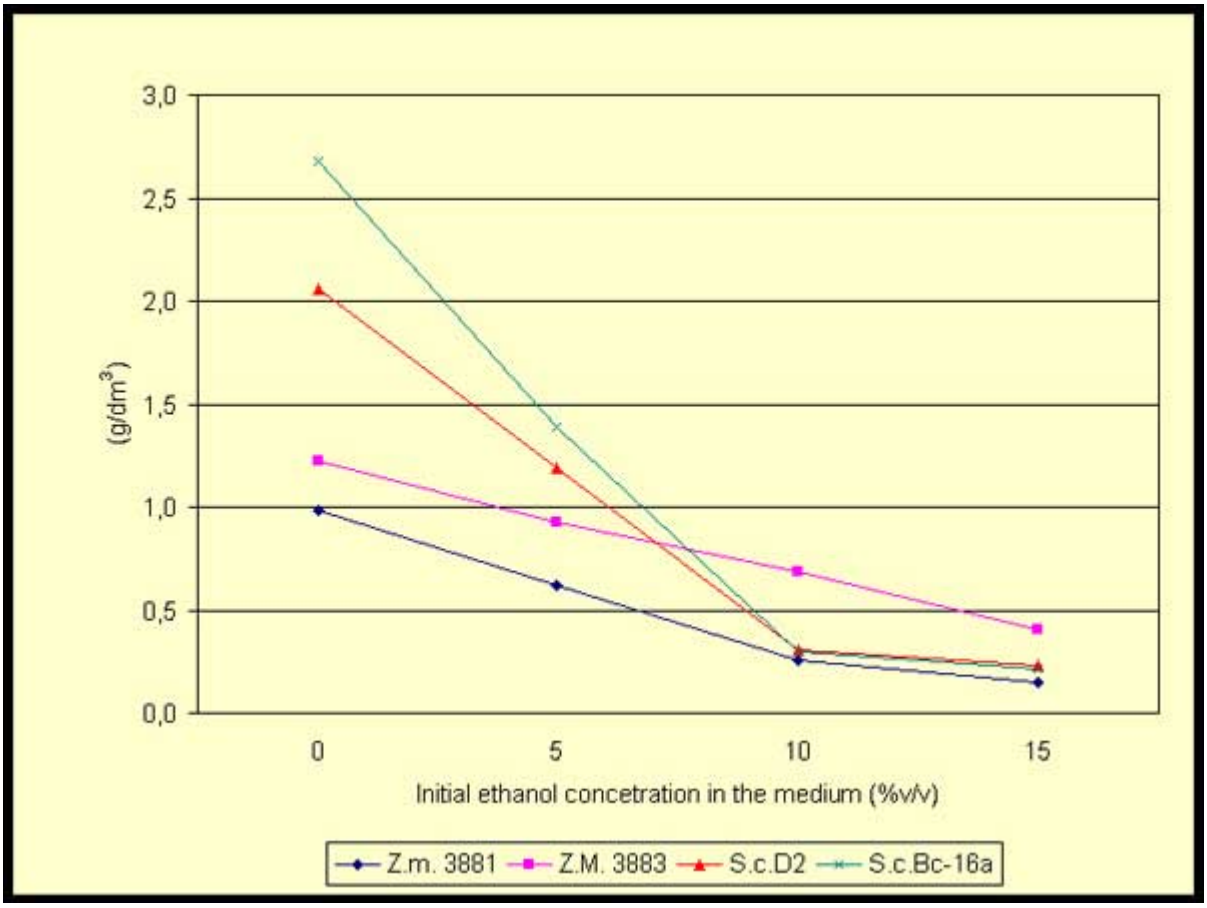
Fig. 2. The amount of bacteria and yeast biomass necessary to produce 1 dcm^3 of 100% ethanol on media with different levels of glucose in the medium



Accumulation of alcohol during fermentation is accompanied by a progressive decrease of rate of sugar conversion to ethanol and repress microorganisms growth. The mechanism of this is not fully recognized there is evidence that inhibition of fermentation can be attributed to an indirect effect of ethanol and by repression of the enzymes of glycolysis involving the plasma membrane which allow leakage of essential cofactors and coenzymes (Doelle *et al.*1993, Osman and Ingram 1985, Hobley and Pammet 1994).

In this work the attempt just to estimate the growing possibilities of used strains of bacteria and yeast in the present of ethanol in the medium was made. The test was planned to compare the amount of biomass formed in standard medium (100 g/dcm^3 glucose) connected 0, 5, 10 and 15% (v/v) ethanol in the beginning of fermentation. The addition of ethanol to the medium influenced yeast growth more than bacteria (Fig. 3). Still 5% ethanol concentration in the medium partly inhibited growth of both types of microorganisms and 15% concentration nearly fully inhibit the growth of used ethanologens. From the four strains tested (two strains of *Zymomonas* and two strains of yeasts), *Z.mobilis* CCM 3883 was the best to adopt to the ethanol presence in the medium.

Fig. 3. Bacteria and yeast biomass production on glucose medium with different ethanol levels



Continuous fermentation of glucose medium (136 g/dcm^3) was confronted using 0.38 dcm^3 working capacity bioreactor with gentle stirring (50 min^{-1}). Two dilution rates was used, one D=0.12 h^{-1} which was thought to be preferable for slower growing yeast cells and the second dilution rate D=0.2 h^{-1} which was close to the maximum growing rate expecting for yeasts. Theoretically the minimum retention time for medium containing glucose (150 g/dcm^3) should not be less than 4-4.5 h [12]. Practically longer retention times are used. Using dilution rate D=0.2 h^{-1} glucose medium is kept in the fermentor for 5 hours. Mixing and slight aeration for yeasts in such a dilution rates are preferable.

The comparison of ethanol content, glucose utilization and ethanol productivity (Fig. 4 and 5) showed that in both dilution rates used tested, *Zymomonas* was significantly better than the used commercial strains of yeasts. Even for the process with lower dilution rate (the retention time 8.33 h) both ethanol yield, and sugar utilization were higher for bacteria and the productivity, which seems to be one of the most important parameter in continuous fermentations, of bacteria was almost twice of that of yeasts (Fig. 4).

Fig. 4. Comparison of bacteria and yeast ethanol production in continuous fermentation (D=0.12 h^{-1}) of glucose medium

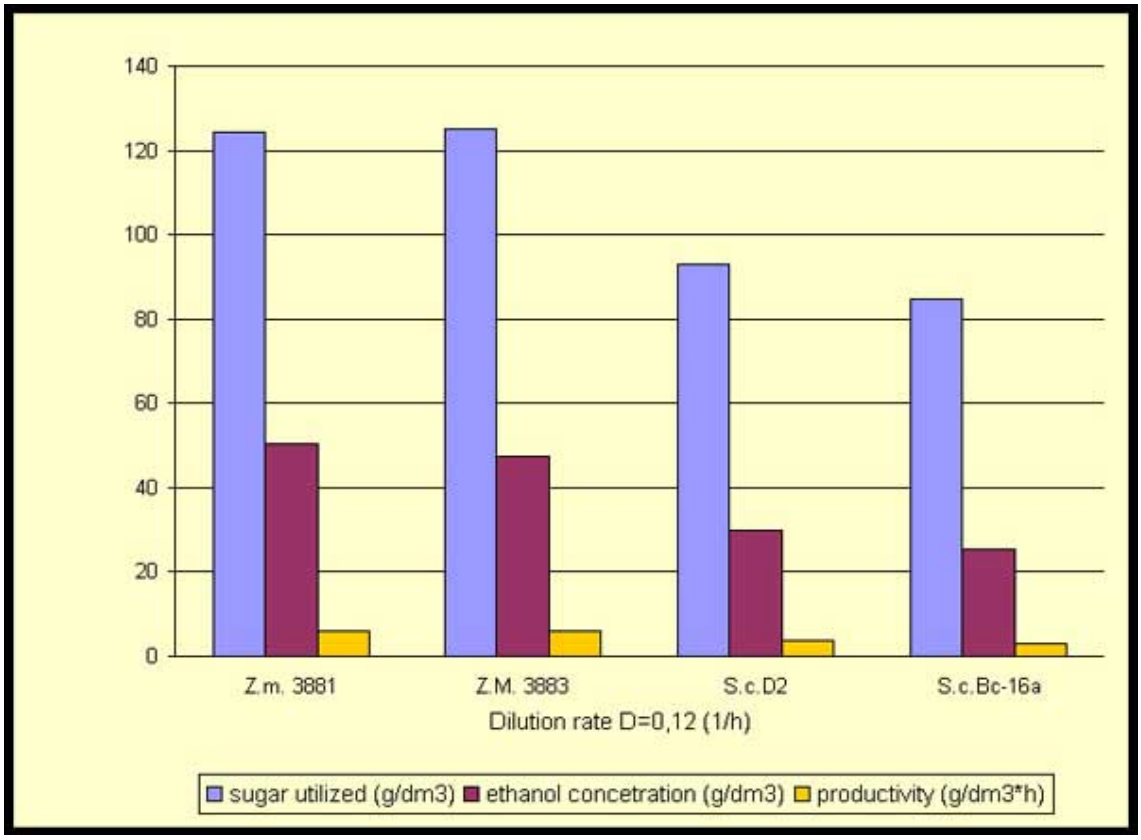
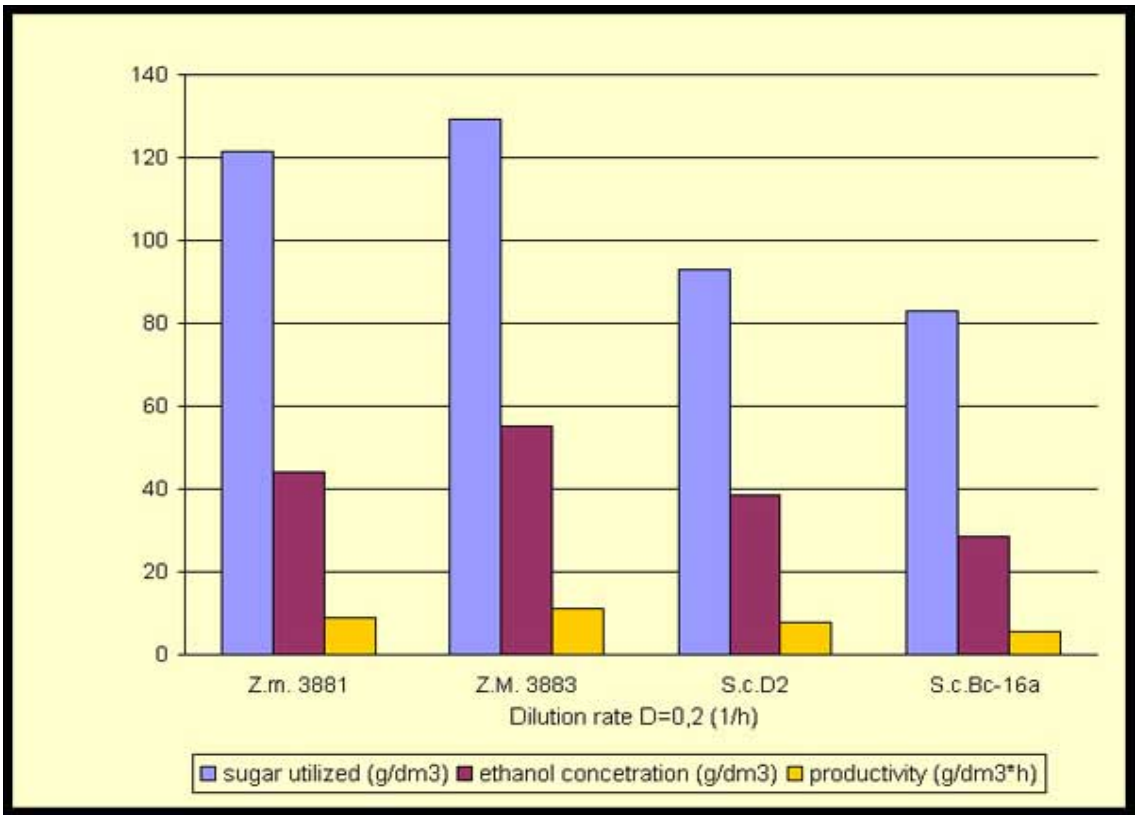


Fig. 5. Comparison of bacteria and yeast ethanol production in continuous fermentation (D=0.2 h^{-1}) of glucose medium



Better results was achieved in D=0.2 h^{-1} when the productivities of yeast (5.68-7.73 g/dcm^{3}· h) were much closer to that of bacteria strains (8.84-11.05 g/dcm^{3}· h). Still the sugar utilization and concentration of produced ethanol was not acceptable. The best results gave *Z.mobilis* 3883. It produced 5.5 g/dcm^3 ethanol, consuming 95.1% of total glucose and achieved the productivity exceeded 11 g/dcm^{3}· h.

The relatively low parameters (but comparable with those cited in the literature - Ishizaki et al. [9]) we linked with high wash out of biomass from bioreactor during fermentation. The amount of yeast biomass coming out with the effluent was as high as 6-7.5 g d.m. in 1 dcm^3 and 3-6 g of dry bacterial bomass in 1 dcm^3. Because of that it was decided to use the immobilization procedure to enhance the biomass concentration in the bioreactor and enhance the productivity.

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

Using the immobilized system higher dilution rates could be applied which markedly increased the productivities of the used bioreactor. When dilution rate D=0.5 h^{-1} employed the ethanol productivities of bacteria settled on the level of 23.8-25.5 g/dcm^{3}· h (yeasts 14.5-15.8 g/dcm^{3}· h) (Fig. 6). Applying D=1.0 h^{-1}, dilution rate relatively for that kind of immobilized fermentor, the ethanol productivity up to 37.0 g/dcm^{3}· h for *Z.mobilis* CCM 3881 and 32.3 g/dcm^3*h for *Z.mobilis* CCM 3883 was achieved. The productivity of yeast was in this fermentation increased much less, reaching 16.8 g/dcm^{3}· h (D_2) and 19.1 g/dcm^3*h for Bc-16a (Fig. 7).

Fig. 6. Comparison of bacteria and yeasts ethanol production capabilities, when immobilized in alginate gel beads, in continuous fermentation (D=0.5 h^{-1}) of glucose medium

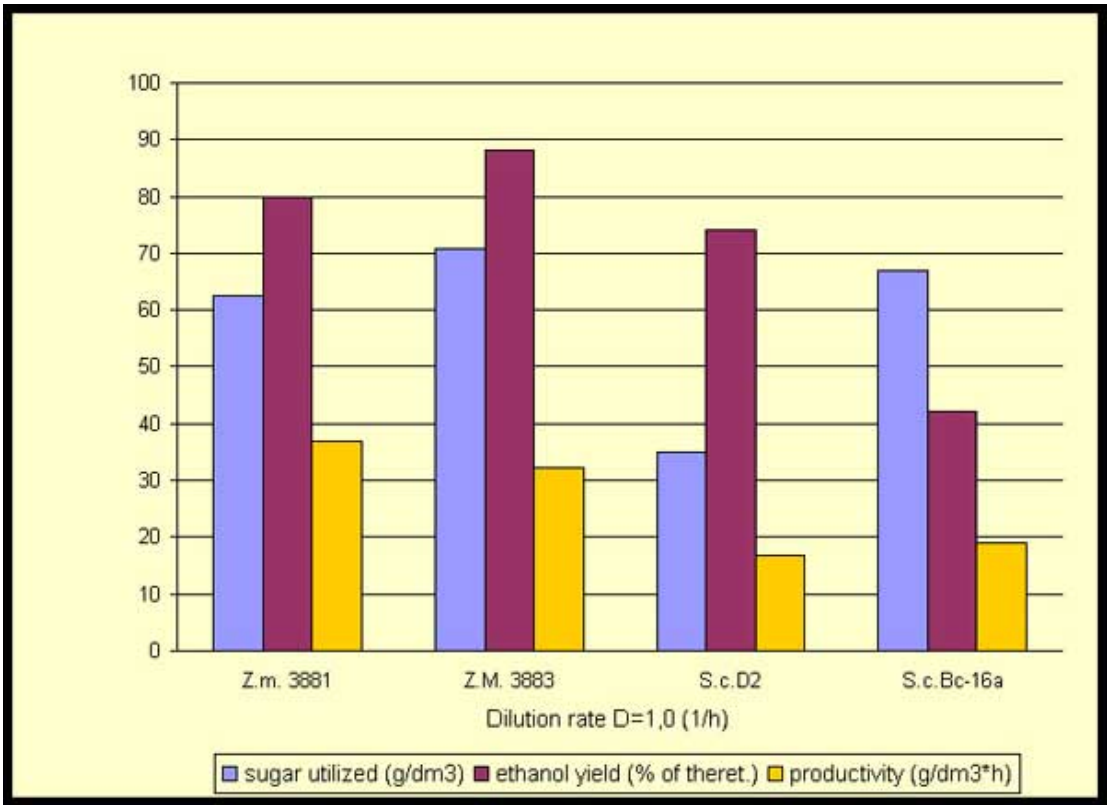
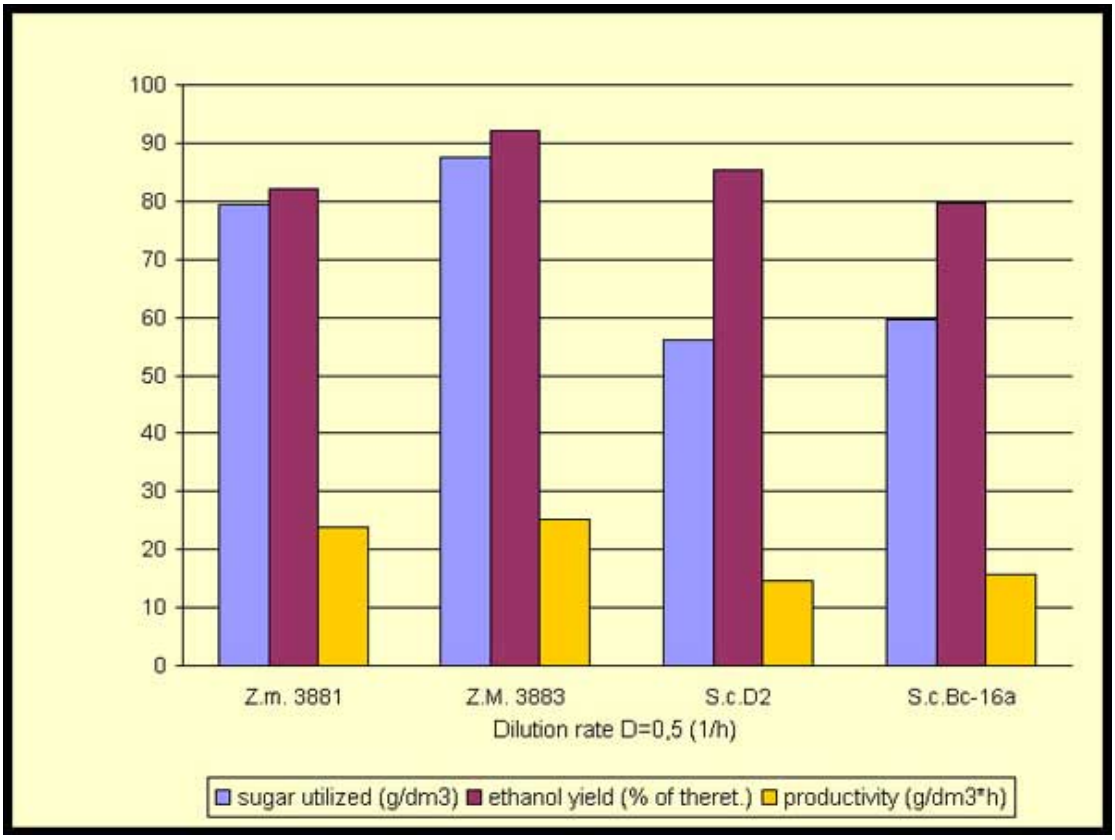


Fig. 7. Comparison of bacteria and yeasts ethanol production capabilities, when immobilized in alginate gel beads, in continuous fermentation (D=1.0 h^{-1}) of glucose mediu



CONCLUSIONS

Comparison of two strains of ethanol producing bacteria *Zymomonas mobilis* and two commercially used in Polish distillery industry yeasts proved that the bacteria represents a high potential possibilities as ethanol producer. The two used strains of bacteria have some advantages against the industrially used yeast as: higher yield when media with high glucose concentration is used, less inhibition by ethanol and higher productivities in continuous fermentation and especially in continuous fermentation when cells were immobilized in alginate beads.

The above mentioned advantages, from the other hand, were demonstrated on rich glucose medium. There is evidence that the situation might be different when industrial media as rye mashes are used (almost 90% of ethanol is produced in Poland from rye). The yield of alcohol produced by tested bacteria are the same as industrial yeasts [15].

However there is to emphasise that bacteria are more suitable for ethanol production, when more dynamic, continuous fermentation techniques are used, especially with immobilized cells.

As it seems that a serious challenge will concern bioethanol producing industry of 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., (1990), Ethanol fermentation by *Zymomonas mobilis* ATCC 10988 in repeated batch cultures. J.Chem.Technol.Biotechnol. 47: 1-6.
2. Buchholz S.F., Eveleigh D.F., (1990), Genetic modification of *Zymomonas mobilis.* Biotechnol. Adv. 8: 547–566.
3. Busche R. M., Scott C. D., Davison B. H., Lynd L. R., (1992), Ethanol, the ultimate feedstock. A technoeconomic evaluation of ethanol manufacture in fluidized bed bioreactors operating with immobilized cells. Appl. Biochem. Biotechnol. 34/35: 395-415.
4. Doelle H.W., Kirk L., Crittenden R., Toh H., Doelle M., (1993), *Zymomonas mobilis* - science and industrial application. Crit. Rev. Biotechnol. 13: 57-98.
5. Dumsday G.J., Jones K., Stanley G.A., Pamment N.B., (1997), Recombinant organisms for ethanol production from hemicellulosic hydrolyzates. Austral. Biotechnol. 5: 285-295.
6. Falcao de Morais J.O., Rios E.M.M., Calazans M., Lopes C.E., (1993), *Zymomonas mobilis* research in the Pernambuco Federal University. J. Biotechol. 31: 75-91.
7. Gałaj M., Pietrzykowska M., Włodarczyk Z., (1994), Preliminary comparison of some substrates ethanol fermentation using *Zymomonas mobilis* bacteria and idustrial yeasts. Przem. Ferm. Owoc. -Warzyw. 9: 13-15.
8. Hobley T.J., Pammet N.B., (1994), Difference in response of *Zymomonas mobilis* and *Saccharomyces cerevisiae* to change in extracellular ethanol concentration. Biotechnol. Bioeng. 43: 155-158.
9. Ishizaki A., Tripetchkul S., Tonokawa M., Zhong-Ping S., Kazuyuki S., (1994), pH mediated control methods for continuous ethanol fermentation using *Zymomonas mobilis*. J. Ferment. Bioeng. 77: 541-547.
10. Karsch T., Stahl U., Esser K., (1983), Ethanol production by *Zymomonas* and *Saccharomyces*, advantages and disadvantages. Eur. J. Appl. Microbiol. Biotechnol. 18: 387-391.
11. Lawford H.G., (1988), A new approach to improving the performance of *Zymomonas* in continuous ethanol fermentation. Appl. Biochem. Biotechnol. 17: 203-209.
12. Luong J.H.T., Tseng M.C., (1984), Process and technoeconomics of ethanol production by immobilized cells. Appl. Microbiol. Biotechnol. 19: 207-216.
13. Miller G.L., (1959), Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31: 426-428.
14. Nowak J., (1998), Genetic modyfication of microorganisms for ethanol production from hemicelluloses. Biotechnologia 3: 89-97.
15. Nowak J., 1999, Bacterium *Zymomonas mobilis* – mikroorganism alternative to the distillery yeasts. Rocz. AR Pozn. PhD Thesis 300, Wyd. AR Poznań.
16. Nowak J., Czarnecki Z., Kamiński E., 1997, The influence of fermentation technique and microorganism on spirits quality. Proc. 1st Symp. In Vino Analytica Scientia, Bordeaux, France 1997, 431-434.
17. Nowak J., Roszyk H., (1997), Co-immobilization of *Aspergillus niger* and *Zymomonas mobilis* for ethanol production from starch. Pol. J. Food Nutr. Sci. 6: 65-70.
18. Oaxaca V.A., Jones L.P., (1991), Formation of ethanol and higher alcohols by immobilized *Zymomonas mobils* in continuos culture. Acta Biotechnol. 11: 523-532.
19. Osman Y.A., Ingram L.O., (1985), Mechanism of ethanol inhibition of fermentation in *Zymomonas mobilis* CP4. J. Bacteriol. 164: 173-180.
20. Queresi N., Manderson G.J., (1995), Bioconversion of renewable resources into ethanol: an economic evaluation of selected hydrolysis, fermentation, and membrane technologies. Energy Sources 17: 241-265.
21. Rios E. M., Salazans G. M. T., Antvnes R. V., Accidy L. G. A., Otamar J., Morais F., (1991), 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.
22. Rogers P.L., Strzelecki A.T., Goodman A.E., (1986), Commercial potential of *Zymomonas* process for ethanol production. Found. Biotechol. Industr. Ferm. Res. 4: 63-79.
23. Struch T., Neuss B., Bringer-Meyer S., Sahm H., (1991), Osmotic adjustment of *Zymomonas mobilis* to concentrated glucose solutions. Appl. Microbiol. Biotechnol. 23: 201-211.
24. Zhang M., Eddy Ch., Deanda K., Finkelstein M., Picataggio S., (1995), Metabolic engineering of pentose metabolism pathway in ethanologenic *Zymomonas mobilis.* Science 267: 240-243.

Submited:

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