Annals of Warsaw University of Life Sciences - SGGW
Forestry and Wood Technology № 91, 2015: 126-133
(Ann. WULS - SGGW, For. and Wood Technol. 91, 2015)

The study of the influence of temperature, nutrient medium and acetate buffer addition on glucose fermentation process

ANDRZEJ ANTCZAK[1], NATALIA SPYSZEWSKA[1], JANUSZ ZAWADZKI[1], MONIKA MARCHWICKA[1], ADAM KUPCZYK[2]

[1]Department of Wood Science and Wood Protection, Warsaw University of Life Sciences – SGGW
[2]Department of Organization, Management and Production Engineering, Warsaw University of Life Sciences – SGGW

Abstract: *The study of the influence of temperature, nutrient medium and acetate buffer addition on glucose fermentation process.* In this paper fermentation process of glucose in various conditions was studied. The aim of the studies was to examine the influence of various factors (temperature, nutrient medium and acetate buffer addition) on glucose fermentation process. In the studies, standard of anhydrous D-glucose was used as a research material. The glucose standard solutions and fermentation mixtures were analyzed by the HPLC with a refractometric detector. Based on the studies, it was found that 0.1% of diammonium hydrogen phosphate addition as a nutrient medium has a positive effect on ethanol production. On the other hand, the acetate buffer (pH = 5) addition has a negative effect. Additionally, it was confirmed that the temperature of fermentation process is a very important factor, which affects the yield of ethanol. After 23 days at 30 °C the production of ethyl alcohol occurs with about twice higher yield than at 25 °C.

Keywords: ethanol, fermentation process, acetate buffer, glucose, HPLC, biofuels

INTRODUCTION

Currently, it is difficult to imagine life without the use of energy. We need it every day, while performing standard activities, for heating and lighting, but also to much more advanced, such as transport or industrial production. The main sources of energy are fossil fuels: oil, coal and natural gas. However, an increase in energy demand, shrinking of resources as well as ecological and economic considerations put before us new challenges in this field. In recent years, many researchers have made the effort to develop effective methods of obtaining energy from renewable sources. One of the attractive and renewable energy is biomass. Plants (trees, shrubs, grasses or perennials), rich in polysaccharides and lignin, can be used to produce of energy and for producing both liquid fuels and gaseous (Lisowski, 2010). Bioethanol is one of the most important fuels obtained from biomass. Currently, the increase of bioethanol global production has been observed. The largest producers of bioethanol are the U.S., Brazil and China (Koh and Ghazoul, 2008). The main raw materials are sugarcane in Brazil and corn in the United States (Kim and Dale, 2004; Jegannathan et al., 2009). In the fermentation process, bioethanol is mainly obtained from glucose (Taherzadeh and Karimi, 2007; Brethauer and Studer, 2014). The yield of the fermentation process depends on many factors. The most important of these include temperature, pH of an environment, an initial concentration of sugar, an amount of nutrient and yeasts (Jarosz and Jarociński, 1994; Chmiel, 1998).

Therefore, the aim of the studies was to examine the influence of various factors (temperature, nutrient medium or acetate buffer addition) on glucose fermentation process and adapt the process for laboratory conditions. The development of appropriate fermentation procedure is very important in the context of future researches on obtaining ethanol from poplar biomass.

MATERIALS AND METHODS

In the studies, standard of anhydrous D-glucose (Poch S.A., Poland) was used as a research material. The process of ethanol obtaining was performed at different temperatures (25 and 30 °C), with or without addition of nutrient medium, and with or without addition of

buffer (pH = 5). Diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) (Chempur, Poland) was used as a nutrient medium, and the buffer was a mixture consisting of 0.2 M sodium acetate solution (CH_3COONa) (Chempur, Poland) and 0.2 M solution of acetic acid (CH_3COOH) (Chempur, Poland).

I fermentation process – 25 °C and nutrient medium addition ($(NH_4)_2HPO_4$)

Fermentation was carried out in four conical flasks (300 cm^3), in which an approximately 25 g of standard glucose and 50 cm^3 of distilled water were placed. The content of each flask was stirred until the sugar was completely dissolved. Then, about 35 mg of dried FERMIOL distillery yeasts (*Saccharomyces cerevisiae*) were added to each flask. Moreover, a different amount (0.1; 1 and 10%) of nutrient medium to three of them was placed. To one of the flasks, the nutrient medium was not added. Finally, the all flasks were poured with 50 cm^3 of distilled water. The flasks were sealed with stoppers provided with fermentation tubes. Fermentation was carried out in a water bath at 25 °C. During the process, samples (1.5 cm^3) were taken. Then, the samples were filtered through 0.45 mm PTFE disks. Finally, 0.1 cm^3 of a sodium azide (NaN_3) (Poch S.A., Poland) solution was added in order to protect the samples against growth of undesirable microorganisms. The samples, which were taken prior to HPLC (High Performance Liquid Chromatography) analysis, were kept in a refrigerator at 6 °C. The duration of the fermentation process was 28 days. The pH measurement of the mixture was performed before and after the fermentation process. For this purpose the microcomputer pH meter CP - 551 equipped with IJ 44C electrode (Elmetron, Poland) was used.

II fermentation process – 30 °C and buffer addition (CH_3COOH + CH_3COONa)

Fermentation was carried out in two conical flasks (300 cm^3), in which an approx. 25 g of standard glucose was weighed. Then, 50 cm^3 of distilled water was added to one of them, and 50 cm^3 of buffer (CH_3COOH + CH_3COONa) (pH = 5) to the other flask. The content of each flask was stirred until the sugar was completely dissolved. Subsequently, 35 mg of dried FERMIOL distillery yeasts (*Saccharomyces cerevisiae*) and 0.1% of nutrient medium were added to the both flasks. Then again, 50 cm^3 of distilled water and 50 cm^3 of buffer were placed in the appropriate flasks. The flasks were sealed with stoppers provided with fermentation tubes. Fermentation was carried out in a water bath at 30 °C. During the process, samples (1.5 cm^3) were taken. Then, the samples were filtered through 0.45 mm PTFE disks. Finally, 0.1 cm^3 of NaN_3 solution was added. The samples, which were taken prior to HPLC analysis, were kept in a refrigerator at 6 °C. The duration of the fermentation process was 23 days. The pH measurement of the mixture was performed before and after the fermentation process. For this purpose (as earlier) the microcomputer pH meter CP - 551 equipped with IJ 44C electrode (Elmetron, Poland) was used.

HPLC analysis

HPLC analysis was carried out using a chromatograph (Shimadzu company) equipped with oven (CTO - 20A, Shimadzu), refractive index detector (RID - 10A, Shimadzu), pump (LC - 20AD, Shimadzu), degasser (DGU-20A, Shimadzu) and control module (CBM-20A, Shimadzu). The results were processed using the software LC Solution v.1.21 SP1. Supelcogel column C - 610H (Supelco® company) with dimensions 300 × 7.8 mm was used to analysis of glucose standard solutions and fermentation mixtures. Analysis conditions were as follows: 0.005M H_2SO_4 (Chempur, Poland) as the eluent, oven temperature: 60 °C, eluent flow: 1.2 cm^3/min, and sample volume: 20 μL.

The calibration curves of ethanol and glucose were prepared to quantitative determination of these substances in a fermentation mixture. The preparation method of

ethanol standard solutions was as follows: concentrated ethanol solution was prepared in a 25 cm^3 volumetric flask (0.5 cm^3 of 96% ethanol (Poch S.A., Poland) and supplementation with distilled water to the mark). Then, the ethanol concentrated solution (15.48 mg/cm^3) was used to obtain diluted solutions of different concentrations (10.32; 7.74; 5.16; 3.10 and 1.41 mg/cm^3). Glucose standard solutions were prepared similarly. The 1.0059 g of glucose in a 100 cm^3 volumetric flask was weighed, 50 cm^3 of distilled water was added and after the sugar dissolution total volume of flask was made up to the mark with distilled water. Then, the glucose concentrated solution (10.06 mg/cm^3) was used to obtain diluted solutions of different concentrations (6.71; 5.03; 3.35 and 2.01 mg/cm^3). Equations of ethanol and glucose calibration curves are presented below:

$y=563x$; $R^2=1.0000$ (ethanol)
$y=1331x$; $R^2=0.9999$ (glucose)

RESULTS AND DISCUSSION

One of the investigated factors which affect the efficiency of fermentation process is an addition of nutrient medium. The results of ethanol concentration after fermentation process with different amount of nutrient are presented in Fig. 1.

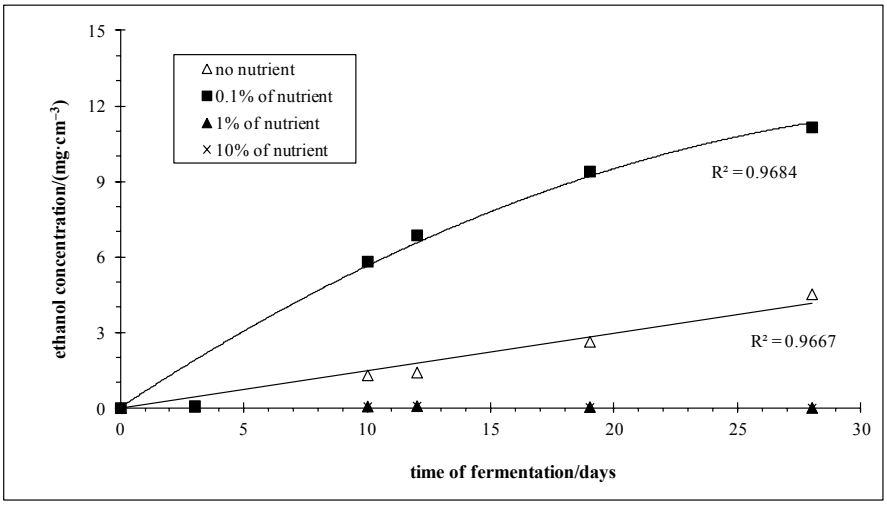

Fig. 1. The ethanol concentration after fermentation process with different amount of nutrient

In the fermentation which was conducted with 1 and 10% of the nutrient medium addition the ethanol was not observed (Fig. 1). Probably the reason for this was too high pH of the fermentation mixture, which stopped a growth of yeasts and did impossible to start the fermentation process. The measured pH of mixtures with 1 and 10% of nutrient medium addition before fermentation were respectively 8.14 and 8.61 (Table 1). The measured values of pH for these mixtures after fermentation decreased and were respectively 6.66 and 6.84 (Table 1). The optimal pH for development of yeasts *Saccharomyces cerevisiae* ranges from 4.5 to 5.5 (Chmiel, 1998). In turn, relating to fermentation process without and with 0.1% of nutrient addition the ethanol was produced. The highest ethanol concentration was observed in samples with 0.1% of diammonium hydrogen phosphate addition (Fig. 1). In the case of these two fermentations the pH measured at the beginning and at the end of fermentation was close to the optimal and ranged from 4.08 to 6.04 (Table 1).

Table 1. The pH of mixtures before and after fermentation process

Conditions of fermentation	pH	
	before	after
no nutrient; 25 °C	5.83	4.08
0.1% of nutrient; 25 °C	6.04	4.23
1% of nutrient; 25 °C	8.14	6.66
10% of nutrient; 25 °C	8.61	6.84
buffer; 0.1% of nutrient; 30 °C	5.00	5.06

The chromatographic analysis of mixtures from fermentation, which was carried out at 30 °C, showed that the applied conditions have a significant impact on the process and its results. The use of acetate buffer, which is intended to maintain a constant optimal pH = 5 in the mixture, had a negative effect on the production of alcohol. Although the pH of the fermentation mixture was maintained at a level of 5.06 (Table 1), it turned out that in a sample taken on the last day of fermentation practically no signal was observed from ethanol (Fig. 2).

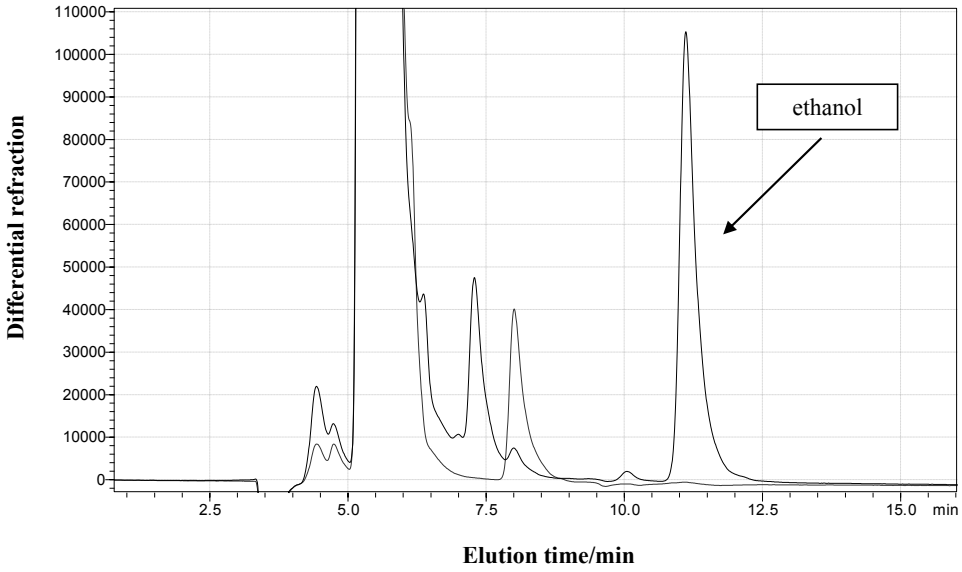

Fig. 2. Chromatograms from analysis of fermentation mixture on Supelcogel C610 – H column; black colour (23 days of fermentation, 30 °C, 0.1% of nutrient medium, no buffer), blue colour (23 days of fermentation, 30 °C, 0.1% of nutrient medium, buffer: CH_3COOH + CH_3COONa)

The above problems were also described by other scientists. Sreenath and Jeffries (2000) observed that in the presence of acetic acid fermentation of wood hydrolyzate is inhibited. Moreover, Olsson and Hahn-Hägerdal (1996) claimed that the inhibitory effect of

acetic acid is pH dependent. In turn, Herrero et al. (1985) stated that the above problems with acetic acid are connected with its undissociated form. For comparison, in the second fermentation mixture where there was no an acetate buffer, the chromatographic analysis showed the presence of ethanol as evidenced by the high and sharp peak in Fig. 2.

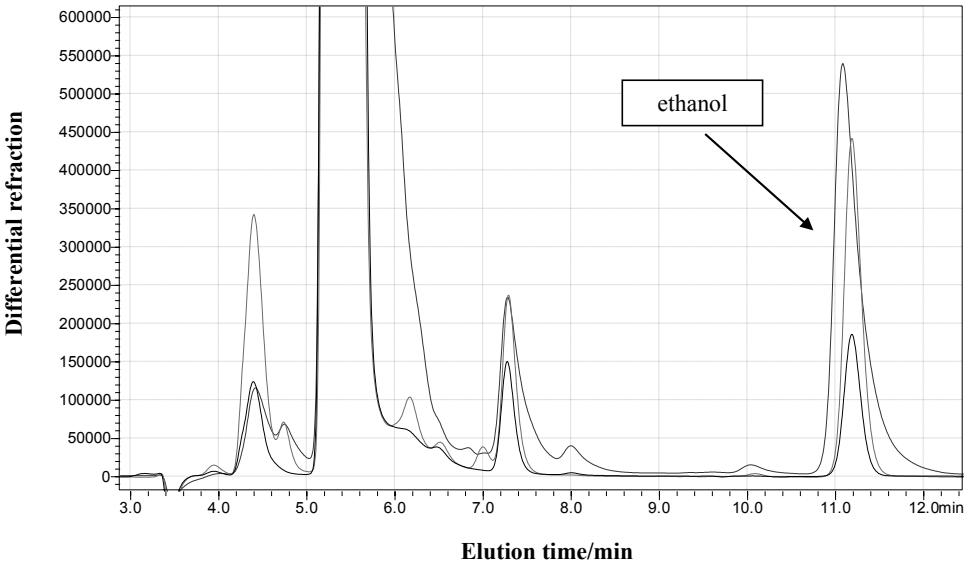

Fig. 3. Chromatograms from analysis of fermentation mixture on Supelcogel C610 – H column; black colour (28 days of fermentation, 25 °C, no nutrient medium, no buffer), red colour (28 days of fermentation, 25 °C, 0.1% of nutrient medium, no buffer), blue colour (23 days of fermentation, 30 °C, 0.1% of nutrient medium, no buffer)

In Fig. 3 the chromatograms from fermentation of glucose were also presented. The fermentation of glucose was carried out without or with 0.1% of nutrient addition and at different temperatures. Fig. 3 shows clearly that the use of 0.1% of nutrient addition and temperature change to a higher have a positive effect on the ethanol formation. At 30 °C the production of ethyl alcohol occurs with high yield and after 23 days the amount of ethanol was higher than after 28 days at 25 °C.

Fig. 4 shows the chromatograms from analysis of fermentation mixture. Duration of the fermentation process was 2, 5, 8, 15 and 23 days. The chromatograms indicate that the process of ethanol production was not begun efficiently from the first days. The peak of ethanol occurred only during the analysis of a sample taken after 8 days of fermentation. This means that despite the favorable conditions alcohol was not obtained. Probably during the first days of fermentation a growth of yeasts took place. After 15 and 23 days the increase of alcohol concentration was observed. To accelerate the start of fermentation process and improve its yield the activation method of yeast should be considered. In addition, the influence of an initial concentration of sugar (glucose) on the fermentation should be checked.

Comparing the results, which were shown in Fig. 5, it can be seen that a change of fermentation conditions has a large effect on ethanol concentration. The rise of temperature it results an increase of the efficiency of the fermentation process. The use of acetate buffer does not give the desired results, and alcohol was not obtained despite the fact that fermentation was conducted at 30 °C. In order to obtain better results, the selection of appropriate buffer should be considered. Perhaps, much better results can be obtained when a mixture of citric acid and sodium citrate will be used.

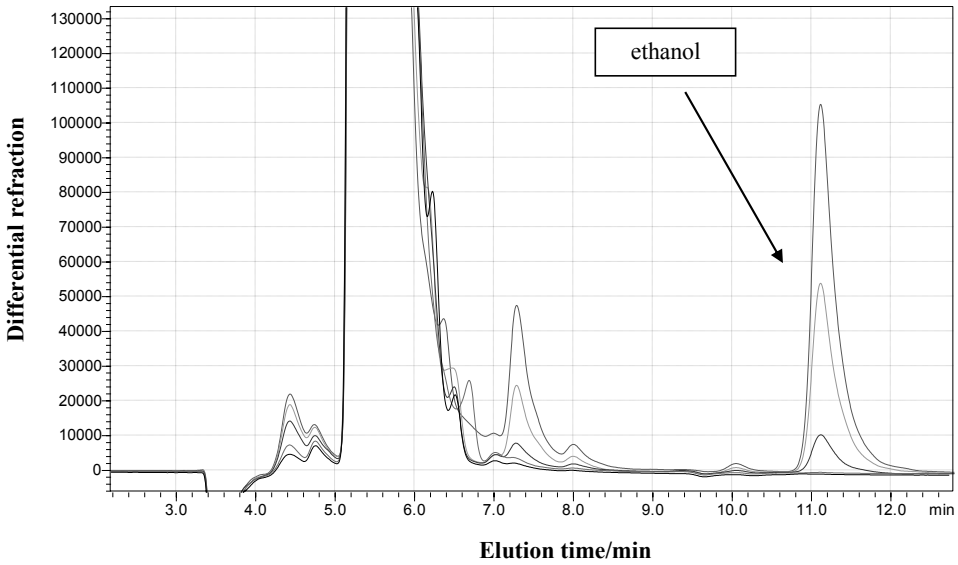

Fig. 4. Chromatograms from analysis of fermentation mixture on Supelcogel C610 – H column; 30 °C, 0.1% of nutrient medium, no buffer; black colour (2 days of fermentation), red colour (5 days of fermentation), blue colour (8 days of fermentation), pink colour (15 days of fermentation), green colour (23 days of fermentation)

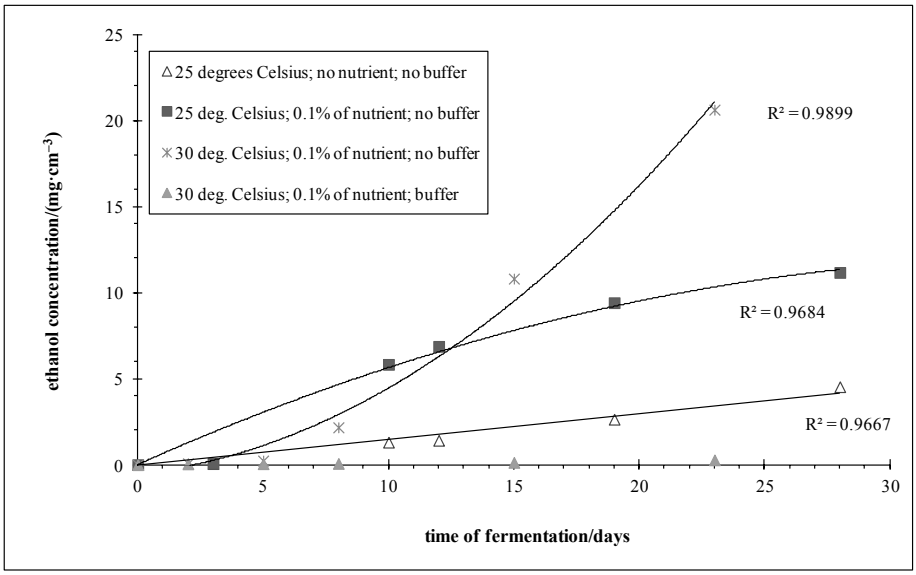

Fig. 5. The ethanol concentration after fermentation process in different conditions

Fig. 6 shows that the loss of glucose during whole time of experiment was quite small (70 mg/cm^3) and also the ethanol concentration on the end of the process reached a value of about only 20 mg/cm^3. These findings confirm the need to elaborate a suitable method of

activation (inoculation) of yeast. The acceleration of yeast growth stage may contribute to more rapid glucose consumption and then the ethanol production increase.

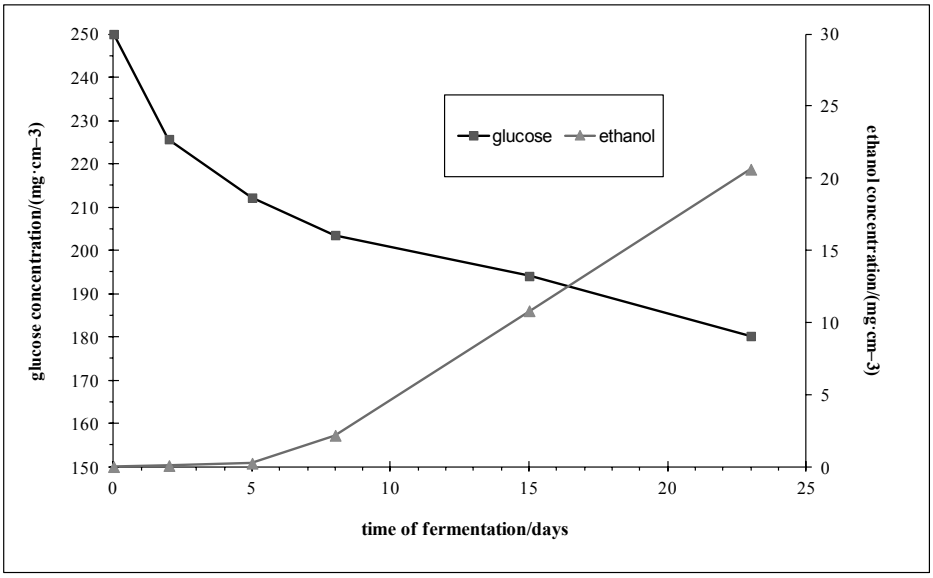

Fig. 6. The glucose and ethanol concentrations after fermentation process; conditions: 23 days of fermentation, 30 °C, 0.1% of nutrient medium, no buffer

CONCLUSIONS

In this paper fermentation process of glucose in various conditions was studied. On the basis of performed experiments the following conclusions were drawn:

1. The addition of a nutrient medium (diammonium hydrogen phosphate) affects the pH of a fermentation mixture. When the nutrient medium addition was too high (1 or 10%), then the growth of yeasts was stopped and did impossible to start the fermentation process. The positive effect on ethanol production was observed in samples with 0.1% of diammonium hydrogen phosphate addition.
2. The acetate buffer (pH = 5) has a negative effect on the production of ethanol. In order to obtain better results, the selection of appropriate buffer should be considered. Perhaps, much better results can be obtained when a mixture of citric acid and sodium citrate will be used.
3. The temperature affects the yield of ethanol. After 23 days at 30 °C the production of ethyl alcohol occurs with about twice higher yield than at 25 °C.
4. The chromatographic analysis indicates that the process of ethanol production was not begun efficiently from the first days. To accelerate the start of the fermentation process and improve its yield the activation method of the yeast should be considered. In addition, the influence of an initial concentration of sugar (glucose) on the fermentation should be checked.

REFERENCES

1.BRETHAUER S., STUDER M. H., 2014: Consolidated bioprocessing of lignocellulose by a microbial consortium, Energy Environ. Sci., 7; 1446-1453.
2.CHMIEL A., 1998: Biotechnologia, Podstawy mikrobiologiczne i biochemiczne, PWN, Warszawa.
3.HERRERO A. A., GOMEZ R. F., SNEDECOR B., TOLMAN C. J., ROBERTS M. F., 1985: Growth inhibition of *Clostridium thermocellum* by carboxylicacids. A mechanism based onuncoupling by weak acids, Appl. Microbiol. Biotechnol., 22; 53-62.
4.JAROSZ K., JAROCIŃSKI J., 1994: Gorzelnictwo i drożdżownictwo, WSiP, Warszawa.
5.JEGANNATHAN K. R., CHAN ENG-SENG, RAVINDRA P., 2009: Harnessing biofuels. A global Renaissance in energy production? Renew. Sust. Energ. Rev., 13; 2163-2168.
6.KIM S., DALE B. E., 2004: Global potential bioethanol production from wasted crops and crop residues, Biomass Bioenerg., 26(4); 361-375.
7.KOH L.P., GHAZOUL J., 2008: Biofuels, biodiversity, and people. Understanding the conflicts and finding opportunities, Biol. Conserv., 141(10); 2450-2460.
8.LISOWSKI A., 2010: Technologie zbioru roślin energetycznych, SGGW, Warszawa.
9.OLSSON L., HAHN-HÄGERDAL B., 1996: Fermentation of lignocellulosic hydrolysates for ethanol production, Enzyme Microb. Tech., 18(5); 312-331.
10.SREENATH H.K., JEFFRIES T.W., 2000: Production of ethanol from wood hydrolyzate by yeasts, Bioresource Technol., 72(3); 253-260.
11.TAHERZADEH M.J., KARIMI K., 2007: Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: a review, BioResources, 2(4); 707-738.

Streszczenie: *Badanie wpływu temperatury, dodatku pożywki i buforu octanowego na proces fermentacji glukozy*. W pracy zbadano przebieg procesu fermentacji glukozy w różnych warunkach. Celem badań było sprawdzenie wpływu różnych czynników (temperatury, dodatku pożywki i buforu octanowego) na przebieg procesu fermentacji glukozy. Jako materiał badawczy wykorzystano bezwodną, wzorcową D-glukozę. Roztwory wzorcowej glukozy i otrzymane mieszaniny po procesie fermentacji poddano analizie przy użyciu metody HPLC z detektorem refraktometrycznym. Na podstawie wyników zaobserwowano, że dodatek pożywki, wodorofosforanu diamonu w ilości 0,1%, posiada korzystny wpływ na otrzymywanie etanolu. Z drugiej strony dodatek buforu octanowego (pH=5) posiada negatywny wpływ na produkcję etanolu. Dodatkowo potwierdzono, że temperatura procesu fermentacji jest bardzo ważnym czynnikiem, który wpływa na wydajność etanolu. Okazało się, że po 23 dniach w 30 °C proces otrzymywania alkoholu etylowego przebiega z około dwukrotnie większą wydajnością niż w temperaturze 25 °C.

Corresponding author:

Andrzej Antczak
Department of Wood Science and Wood Protection,
Warsaw University of Life Sciences (SGGW)
ul. Nowoursynowska 159, 02-776 Warsaw, Poland
e-mail: andrzej_antczak@sggw.pl