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THE OCCURRENCE AND IDENTIFICATION OF MICROBIOLOGICAL CONTAMINATION IN FUEL ETHANOL PRODUCTION*

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Background. Bacterial contamination is a major problem for commercial fuel ethanol production in distilleries all over the world. The contaminating microorganisms produce acetic and lactic acid that has a detrimental effect on fermentation efficiency. The aim of this work was to calculate the number of bacterial contaminants in some distilleries. Moreover, in this study it was signified what kind of bacteria contaminate ethanol production process.
Material and methods. Grains were obtained from five distilleries from some regions in Poland, in this work hereafter referred to as α, β, γ, δ, and ε distilleries. Corn was the raw material in the α, β, and γ distilleries, triticale in δ distillery, and rye in the ε one. From these five distilleries, sweet mashes during fermentation and after it, were also analysed. The total number of microorganisms, the number of lactic acid bacteria, the number of anaerobic bacteria and the quantity of yeasts and moulds in raw materials were calculated.
Results. The number of total viable bacteria (CFU/g), lactic acid bacteria (CFU/g), anaerobic bacteria (CFU/g), moulds, and yeasts (CFU/g) occur in the samples were determined. In all distilleries tested, all groups of microorganism were present.
Conclusions. The results of our study show that all tested distilleries have a lot of difficulties with microbiology pollution which leads to a decrease of ethanol production and economical problems. From the economical point of view, reduction of microbial contamination makes it possible to increase the production volume.

Key words: contaminants, distillery, fuel ethanol production, lactic acid bacteria

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INTRODUCTION

Currently ethanol as transportation fuel is of interest to many factories branches of industry. However, bacterial contamination is an outgoing problem for commercial fuel ethanol factories. In distilleries, contaminants can originate from tankage, transfer lines, heat exchangers, raw materials, active dry yeasts, poorly stored backset, or yeast slurry used as the inoculums [Narendranath et al. 1997, Reed and Nagodawithana 1999]. They can significantly limit the scale of ethanol production from agricultural sources [Schell et al. 2007, Skinner and Leather 2004]. Fuel ethanol is not produced under homogeneous culture conditions; moreover, chronic infections are expected and tolerated, although, they are frequently deleterious to the ethanol production process. Infections can lead to stuck fermentation, and bioreactors must be shut down for cleaning. Thus, they decrease the efficiency of ethanol production and increase its costs. Contaminants reduce carbon available for conversion to ethanol. Simultaneously, they compete for nutrient factors needed by yeast cells and can produce toxic byproducts (lactic and acetic acids). The primary bacterial contaminants of fuel ethanol fermentations are lactic acid bacteria (LAB) [Skinner-Nemec et al. 2007]. LAB are the most troublesome because of their tolerance to high temperature and low pH, and their ability to grow rapidly [Narendranath et al. 1997]. Removal of contaminating bacteria reduces the microbial competition for nutrients in the growth media thereby increasing the efficiency and productivity of the culture.

Microbial numbers can be significantly reduced by cleaning and sanitizing the equipment, by maintaining backset at a temperature over 70°C, by pasteurizing or chemically sterilizing the substrates [Narendranath et al. 1997]. Furthermore, various agents for control of bacterial contaminants under laboratory conditions have so far been tested: potassium metabisulfite, hydrogen peroxide, 3,4,4'-trichlorocarbanilide, and antibiotics such as tetracycline, penicillin, monensin, and virginiamycin. All of the above-mentioned agents inhibited bacteria over yeasts. Nowadays, penicillin and virginiamycin commercially to inhibit bacterial infections of fuel ethanol production are sold [Skinner and Leather 2004, Gibbons and Westby 1996, Narendranath et al. 2000, Oliva-Neto and Yokoya 1998, Stroppa et al. 2000].

In this paper, the microbiological situation of five distilleries in Poland was discussed. The number of total bacterial contaminants was calculated, as well as the lactic acid bacteria, anaerobic bacteria, moulds and yeasts. The aim of this study was also to specify what kinds of bacteria contaminate ethanol production process.

MATERIAL AND METHODS

The microbiological purity of grains used in fuel ethanol production was tested. Grains were obtained from five distilleries in Poland, in this work hereafter referred to as α, β, γ, δ, and ε distilleries. Corn was the raw material in the α, β, and γ distilleries, while triticale in the δ distillery, and rye in the ε one. Probes of sweet mashes during fermentation and after it were also analysed. Fermentation went on for three days. Samples were taken in the 2008/2009 campaign. Bacteria were cultured in MRS Medium, Lab-Agar, Thioglycollate Fluid Medium, and in Chloramphenicol Lab-Agar (BIOCORP company).

Grains obtained from the distilleries were immersed in physiological salt and shaken out for two hours. The total number of microorganisms, the number of lactic acid bacteria, the number of anaerobic bacteria and the quantity of yeasts and moulds in raw materials were calculated. The inoculations of microorganisms into a medium were done. In case of mash, cykloheximide 100 mg/ml (Sigma) was added to medium to kill yeasts and make it possible to count live bacteria cells.

Selected bacterial isolates were identified by comparing ribosomal RNA gene sequences to known sequences. A 1.6 kb segment of the 16S rDNA was amplified from genomic DNA of a single colony per reaction using bacterial primers. A cycle sequencing kit was used to sequence the amplified product. The nucleotide sequence was obtained from both ends of the PCR product. On the basis of 16s RNA the affinity of the microorganism was determined.

RESULTS AND DISCUSSION

Samples of raw materials (corn, triticale, and rye) were obtained from tested distilleries in February and in March 2009. Grains in all the distilleries were stored in the containers at the room temperature. Purity of these grains was controlled. The number of total viable bacteria (CFU/g), lactic acid bacteria (CFU/g), anaerobic bacteria (CFU/g), moulds, and yeasts (CFU/g) occur in the samples were determined. In all distilleries tested, all the above-mentioned groups of microorganism were present. The level of total viable, lactic acid, and anaerobic bacteria was similar in the β and γ distilleries (about $7 \cdot 10^7$ CFU/g). It was higher than the estimated value (e.g., the number of cereal grains total viable bacteria is assessed at $5 \cdot 10^4$-$1.6 \cdot 10^6$ CFU/g) [Maciorowski et al. 2007]. Both distilleries use corn as the raw material. Corn was also used in the α distillery but in spite of this the level of bacterial contaminants in grains here was lower than in the β and γ distilleries. It may be a result of different storage conditions of corn in these three distilleries. The level of contamination with total viable bacteria (about 10^6 CFU/ml) and lactic acid bacteria (about 10^5 CFU/ml) in the α distillery was similar to the δ distillery. The lowest number of the total viable, lactic acid, and anaerobic bacteria was observed in the material from the ε distillery and was comparable with the estimated value for this kind of grain (bacterial number for wheat reaches 10^5-10^6 CFU/g) [Obuchowski and Strybe 2001]. In this distillery rye is the raw material to fuel ethanol production. And the lowest level of moulds and yeasts was in the β distillery. The contamination by moulds and yeasts was generally on the lowest level in all five distilleries as compared to the other contaminants. It was about 10^4 CFU/ml in the α, γ, δ, and ε distillery and about 10^4 CFU/ml in the β distillery. The detailed data about bacterial contamination are presented in Table 1.

The level of bacteria contaminants in sweet mash at the beginning of the fermentation, in the sweet mash after 24 hours and after complete fermentation in all tested distilleries is presented in Figure 1.

In the α distillery the highest level of total viable and lactic acid bacteria was observed in sweet mash after 24 hours of the fermentation. Also the level of anaerobic bacteria increased after 24 hours of fermentation and was the same up to the end of this process. The number of mould and yeast cells was the same in sweet mash at the beginning of the fermentation and after 24 hours. Decrease to zero was observed only just

Table 1. Microbiological purity of raw materials from five Wielkopolska distilleries

Materials	α	β	γ	δ	Ɛ
Total viable bacteria	6.39	7.60	7.39	6.08	4.75
Lactic acid bacteria	5.69	7.87	7.17	5.50	4.00
Anaerobic bacteria	6.53	7.81	7.17	5.00	4.20
Moulds and yeasts	4.08	3.74	4.53	4.79	4.68

The raw material from the α, β and γ distilleries was corn, triticale from the δ distillery and rye from the Ɛ one.

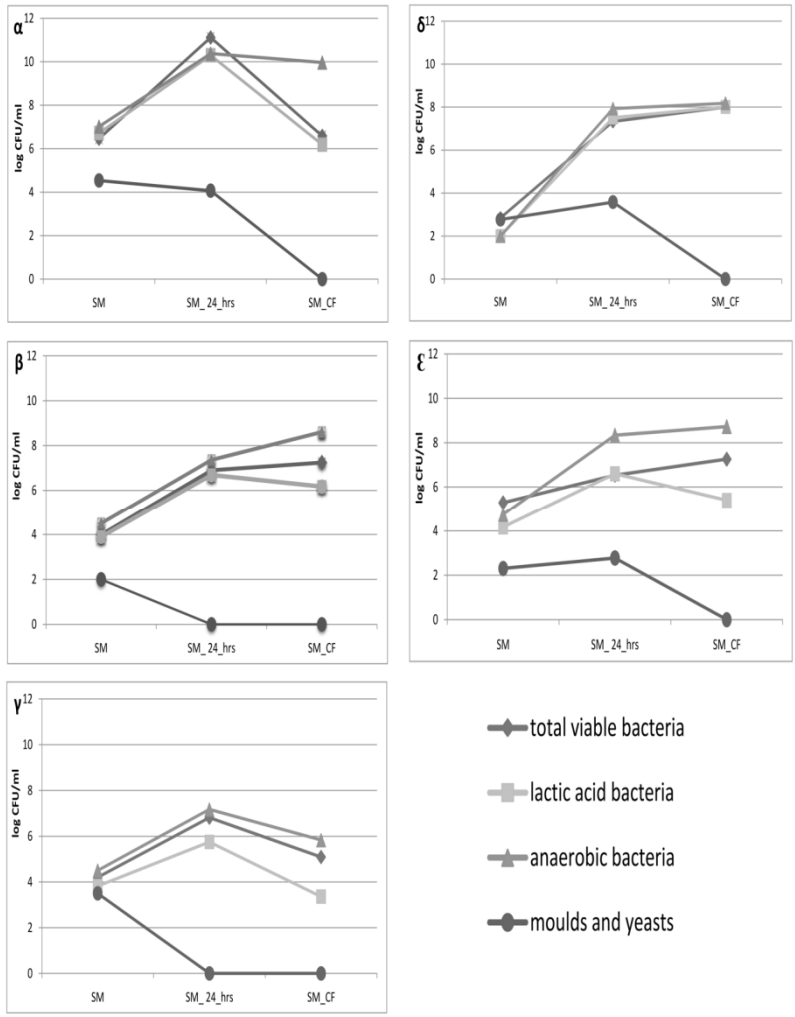

Fig. 1. The number of bacteria contaminants in five distilleries from Poland. Abbreviations: SM – sweet mash, SM_24_hrs – sweet mash after 24 hours of the fermentation, SM_CF – sweet mash after complete fermentation

on the end of the fermentation. It is connected with run-down of oxygen – moulds and yeasts need oxygen to grow and live. In the β distillery all groups of microorganisms, in spite of moulds and yeasts, increased through the first 24 hours of the fermentation. The level of this increase was lower than in the α distillery. After 24 hours of fermentation there was no moulds and yeasts. In the *γ* distillery situation was similar to the α one. However, the number of bacteria contaminants in this distillery was lower. There were no mould and yeast cells after 24 hours of fermentation yet (like in the β distillery). All three distilleries used the same raw material – a corn.

In the δ distillery, the number of total viable, lactic acid, and anaerobic bacteria increased during the first 24 hours of fermentation process. The number of bacteria contaminants in the sweet mash was twice as big as lower than in the α distillery, but after 24 hours it was almost the same in both distilleries. After the complete fermentation no changes were observed. The number of moulds and yeasts increased after first 24 hours of fermentation, and decreased at the end of the process. As similar situation was observed in the Ɛ distillery – the number of moulds and yeasts increased at first and decreased to zero at the end of fermentation. The number of total viable and anaerobic bacteria cells increased after first 24 hours of fermentation and at the beginning of the fermentation. The level of anaerobic bacteria was the highest here because the fermentation is an anaerobic process. Lactic acid bacteria numbers during first 24 hours of fermentation increased and decreased later – as in the α and *γ* distilleries.

Table 2. Bacterial contaminants in Polish distilleries

Bacterial species	Occurrence of the genus within the samples percentage of total isolates				
	α	β	γ	δ	Ɛ
Bifidobacterium adolescientis	22	25	25	23	22
Bifidobacterium angulatum	14	12	12	10	13
Clostridium aerotolerans	11	9	10	11	10
Lactobacillus acidophilus	8	6	4	6	8
Lactobacillus brevis	2	4	3	0	5
Lactobacillus buchneri	3	5	3	6	6
Lactobacillus casei	2	0	4	4	3
Lactobacillus delbrueckii	10	8	8	10	10
Lactobacillus fermentum	10	9	9	11	9
Lactobacillus lactis	1	2	3	3	5
Lactobacillus paracasei	2	3	2	0	1
Lactobacillus reuteri	3	4	2	2	1
Leuconostoc carnosum	2	2	2	0	1
Pediococcus parvulus	1	3	4	3	2
Unidentified	9	8	9	11	4

The identification of microorganisms in fuel ethanol production was also done. Single-colony isolates were purified and identified from each sample. Results are presented in Table 2. In all distilleries tested, lactic acid bacteria made up the majority of identified isolates (22-25% of total isolates). There were also: *Bifidobacterium*, *Clostridium*, *Leuconostoc*, and *Pediococcus*. The number of these microorganisms was nearly at the same level in all five distilleries.

CONCLUSIONS

The results of our study show that all the tested distilleries have a lot of difficulties with microbiological pollution which leads to a decrease of ethanol production and economical problems. From the economical point of view, reduction of microbial contamination makes it possible to increase the production volume. Moreover, the elimination of undesirable microflora will allow to use different waste raw materials and new energy-saving technologies for more efficient ethanol production.

REFERENCES

Gibbons W.R., Westby C.A., 1996. Use of potassium metabisulfite to control bacterial contaminants during fermentation of fodder beet cubes for fuel ethanol. Biomass 11, 99-113.

Maciorowski K.G., Herrera P., Jones F.T., Pillai S.D., Ricke S.C., 2007. Effects on poultry and livestock of feed contamination with bacteria and fungi. Anim. Feed Sci. Technol. 133, 109--136.

Narendranath N.V., Hynes S.H., Thomas K.C., Ingledev W.M., 1997. Effects of *Lactobacilli* on yeast-catalyzed ethanol fermentations. Appl. Environ. Microbiol. 63, 11, 4158-4163.

Narendranath N.V., Thomas K.C., Ingledev W.M., 2000. Urea hydrogen peroxide reduces the numbers of lactobacilli, nourishes yeast, and leaves no residues in ethanol fermentation. Appl. Environ. Microbiol. 66, 4187-4192.

Obuchowski W., Strybe K., 2004. The effect of grain cleaning and milling in some Polish mills on the level of grain and flour microbial contamination. EJPAU, Food Sci. Technol. 1, 1.

Oliva-Neto P., Yokoya F., 1998. Effect of 3,4,4'-trichlorocarbanilide on growth of lactic acid bacteria contaminants in alcoholic fermentation. Bioresour. Technol. 63, 17.

Reed G., Nagodawithana T.W., 1999. Yeast derived products. In: Yeast technology. Van Nostrand Reinhold New York, 225-256.

Schell D.J., Dowe N., Ibsen K.N., Riley C.J., Ruth M.F., Lumpkin R.E., 2007. Contaminant occurrence, indentification and control in a pilot-scale corn fiber to ethanol conversion process. Bioresour. Technol. 98, 2942-2948.

Skinner K.A., Leather T.D., 2004. Bacterial contaminants of fuel ethanol production. J. Ind. Microbial. Biotechnol. 31, 401-408.

Skinner-Nemec K.A., Nichols N.N., Leathers D., 2007. Biofilm formation by bacterial contaminants of fuel ethanol production. Biotechnol. Lett. 29, 379-383.

Stroppa C.T., Andrietta M.G.S., Andrietta S.R., Steckelberg C., Serra G.E., 2000. Use of penicillin and monensin to control bacterial contamination of Brazilian alcohol fermentation. Int. Sugar J. 102, 78-82.

ZANIECZYSZCZENIA MIKROBIOLOGICZNE PROCESU FERMENTACJI ETANOLOWEJ

Wstęp. Zanieczyszczenia bakteryjne są poważnym problemem w przemysłowej produkcji bioetanolu w gorzelniach na całym świecie. Niepożądane mikroorganizmy produkują kwasy octowy i mlekowy, które znacznie obniżają wydajność fermentacji. Celem pracy było oznaczenie liczebności mikroflory zanieczyszczającej gorzelnie na terenie Polski. Ponadto oznaczono najczęściej występujące gatunki bakterii.
Materiał i metody. Badane ziarno pochodziło z pięciu gorzelni z terenu Polski, które oznaczono jako α, β, γ, δ i ε. Surowcem w gorzelniach α, β i γ była kukurydza, w gorzelni δ – pszenżyto, a w ε – żyto. Analizowano również stan mikrobiologiczny zacierów gorzelniczych pochodzących z badanych gorzelni. Określono ogólną liczbę bakterii, liczebność bakterii mlekowych, beztlenowych oraz pleśni i grzybów w analizowanym materiale.
Wyniki. Ogólna liczba bakterii (jtk/g), liczebność bakterii mlekowych (jtk/g), beztlenowych (jtk/g) oraz pleśni i grzybów (jtk/g) została oznaczona dla surowców i zacierów pochodzących ze wszystkich pięciu badanych gorzelni. Mikroorganizmy z badanych grup występowały we wszystkich surowcach i zacierach.
Wnioski. Wyniki badań wskazują, że problem zanieczyszczenia mikrobiologicznego dotyczy wszystkich badanych polskich gorzelni. Znacznie zmniejsza to wydajność produkcji bioetanolu, prowadząc do strat ekonomicznych.

Słowa kluczowe: zanieczyszczenia, gorzelnie, produkcja bioetanolu, bakterie kwasu mlekowego

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