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Original Research

The Microbiological Situation of Distilleries in Poland

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Abstract

Microbiological contamination is a major problem for commercial fuel ethanol production in distilleries all over the world. Undesirable microorganisms compete with yeasts for nutritional substances; moreover, they produce lactic and acetic acids that harm yeast cells. One of the sources of microbiological pollution in the fermentation process is raw materials (e.g. grains). It is important to find out what kind of microflora contaminate them, and new technologies should be developed to reduce this contamination.

The aim of this work was to determine the total number of mesophilic bacteria, the number of lactic acid bacteria, anaerobic bacteria, moulds, and yeasts that occur in raw materials used in distilleries in Poland's Wielkopolska region. Moreover, the numbers of these microorganisms in the sweet mash, in the sweet mash after 24 hours of fermentation, and after complete fermentation were counted. We decided to check out the microbiological state of raw materials and fermentation mashes because of the low bioethanol efficiency reached in these small ethanol plants.

In all five distilleries, mesophilic bacteria, lactic acid bacteria, anaerobic bacteria, moulds, and yeasts occurred in grains. The level of contamination was relatively high. These groups of microorganisms were also present in the sweet mash, in the sweet mash after 24 hours of fermentation and after complete fermentation. The level of contamination was similar in all distilleries that use the same raw materials, and it was rather high. So the obtained results (relatively high raw material and fermentation mashe microbiological contamination) can explain the low ethanol efficiency found in all tested distilleries.

Keywords: microorganism contamination, distillery, mash, fermentation

Introduction

Microbial infections in industrial fermentation processes are an important problem that concerns distilleries all over the world, regardless of the raw materials they are using. Because of undesirable microflora growth, the efficiency of ethanol production from a raw material unit is lower [1]. Contaminating microorganisms in cultured medium are serious competitors to *Saccharomyces cerevisiae* yeasts. These undesirable organisms, among other bacteria families *Pseudomonadaceae*, *Micrococcaceae*, *Lactobacillaceae*, and *Bacillacae*, and moulds such as *Alternaria*, *Fusarium*, *Helminthosporium,* and *Cladosporium*, use sugars and consume nutrition substances for yeasts [2]. What is more, the *Lactobacillus* cells can use sugars which are not consumed by yeasts (e.g. arabinose) and thus they grow very fast [3]. Bacteria synthesize primary and secondary metabolites and a lot of fermentation by-products that are inhibitors to starter *Saccharomyces cerevisiae* cultures. Common contamination bacteria are *Lactobacillus* spp. that produce lactic and acetic acids, a reason for the low efficiency of ethanol

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Table 1. Cultured media used for microbial analysis.

Analyzed group of microorganisms	Medium	Incubation parameters		Method
		Temperature (ºC)	Time (h)	
Total mesophilic bacteria counts	Nutrient LAB-AGAR with 2% w/v of glucose (BIOCORP). Composition: Peptone 5 g/L, Agar 15 g/L, Beef Extract 3 g/L + Glucose 20 g/L.	37	48-72	Koch plate method
Lactic acid bacteria counts	MRS LAB-AGAR (BIOCORP). Composition: Peptone 10 g/L, Meat Extract 8 g/L, Yeast Extract 4 g/L, Glucose 20 g/L, Sodium Acetate Trihydrate 5 g/L, Tween 80 1g/L, Dipotassium Hydrogen Phosphate 2 g/L, Triammonium Citrate 2 g/L, Magnesium Sulfate 0.2 g/L, Manganese Sulfate 0.05 g/L, Agar 15 g/L.	37	48-72	Koch plate method
Anaerobic bacteria counts	Thioglycollate Fluid Medium (BIOCORP) with 1.5% w/v of Agar (BIOCORP). Composition: Enzymatic Digest of Casein 15 g/L, D-Glucose 5.5 g/L, Sodium Chloride 2.5 g/L, Resazurin 0.001 g/L, L-Cystein 0.5 g/L, Yeast Extract 5 g/L, Sodium Thioglycollate 0.5 g/L, Agar 0.75 g/L + Agar 15 g/L.	37 (anaerobical conditions)	48-72	Koch plate method
Moulds and yeast counts	Chloramphenicol LAB-AGAR (BIOCORP). Composition: Yeast Extract 5 g/L, Glucose 20 g/L, Chloramphenicol 0.1 g/L, Agar 12 g/L.	30	48-72	Koch plate method

production [4, 5]. However, it is impossible to avoid all contaminating organisms in cultured medium, but it is necessary to control their level because repeated infections may lead to fermentation inhibition [6], and it causes huge economic loss. The most popular method of control of bacterial contamination, though not accepted in many countries (e.g. in Poland), is using antibiotics such as penicillin G, streptomycin, tetracycline, virginiamycin, and monensin, or mixtures thereof [7].

Sources of microbiological infections in commercial ethanol production can be raw materials, water used in the process, fermentors and other fermentation, equipment. There are many methods for disinfection of water and fermentation equipment, but raw material decontamination technologies still need improvement to become more effective, universal for all kinds of raw materials, harmless for the environment, and cheap [8].

In Polish cereals the most common contaminants are bacteria belonging to the genera *Pseudomonas*, *Micrococcus*, *Lactobacillus*, and *Bacillus*, and moulds such as *Aspergillus fumigates*, *Aspergillus flavus*, *Penicillum notatum*, and *Penicillum expansum* [9]. It is worth noting that *Lactobacillus* is also the most frequent reason for "stuck fermentation" [10]. In the case of potatoes (*Solanum tuberosum*), *Clostridium* infections are often observed in Poland [11]. With white beets (*Beta vulgaris* ssp. *vulgaris convar. crassa var. Altissima*) as a raw material, there is a risk of infections mainly by *Leuconostoc mesenteroides*.

Agricultural distilleries supply alcohol to the spirit and fuel industries. In the last decade, a huge crisis of this kind of distillery in Poland has been observed, and customers for raw spirits argue that this crisis is due to inconsistent raw material quality. One solution of this problem is in introducing a quality guarantee system, since economical effectiveness of ethanol production depends mostly on microbiological purity of the fermentation process [9]. In 1995 there were 950 agriculture distilleries in Poland. In 2006 there were only 217 active production plants. In the Wielkopolska region, in 2009 there are 50 active agricultural distilleries [12].

The aim of this study was to estimate the microbiological situation of selected agricultural distilleries in the Wielkopolska region that have some troubles with achieving good ethanol efficiency. Microbial contamination of raw materials using for bioethanol production and mash during fermentation was checked out.

Experimental Procedures

Materials

Distillery raw materials such as corn (*Zea mays*), triticale (*Triticosecale*), and rye (*Secale cereale*) were tested. They were obtained from five distilleries from the Wielkopolska region, in this work hereafter referred to as A, B, C, D, and E distilleries.

The raw material from the A, B, and C distilleries was corn. Triticale came from the D distillery and rye from E.

From these five distilleries, sweet mash during and after fermentation were analyzed. Fermentation went for three days. Samples were taken in the 2008/2009 cycle.

Solid selective media (BIOCORP, Warsaw, Poland) for microorganism cultures were used: MRS (for lactic acid bacteria), Lab-Agar (to count total viable aerobic bacteria), Thioglycollate Fluid Medium (for anaerobic bacteria), and Chloramphenicol Lab-Agar (for moulds and yeasts). The composition of these mediums and incubation conditions are shown in Table 1.

Estimation of the Levels of Microbiological Infection of Raw Materials and Mashes

Grains (10 g per sample) and mashes (10 mL per sample) obtained from the Wielkopolska distilleries were transferred into a sterile stomacher bag (Merck). 90 mL of a physiological salt (9 g/L NaCl) was added and mixed for 2 min in a DEVIMIX machine (De Ville Biotechnology, Poland). Tenfold dilutions in the range 10^{-1} to 10^{-10} were made from such prepared samples. The total number of mesophilic bacteria, the number of lactic acid bacteria, the number of anaerobic bacteria and the quantity of yeasts and moulds were analyzed using the Koch plate method. The analyses were carried out on duplicate agar plates. Contamination of the mash (which contains a lot of *Saccharomyces cerevisiae* cells leading the fermentation process) was tested using the above-mentioned media supplemented with cycloheximide 100 mg/mL (SIGMA). Cycloheximide effectively kills eukaryote cells (e.g., yeasts) and makes it possible to count live bacteria (in this case on Chloramphenicol Lab-Agar medium only moulds were counted – most of them are more resistant against cycloheximide than *Saccharomyces cerevisiae* used in the fermentation process [13]).

Bacteria Indentification

From plates with MRS and Lab-Agar media, grown by over 50 bacteria colonies, all single representative colonies were chosen and then propagated on liquid medium for 72 hrs at 37°C (microflora from corn grain and corn mashes). Genomic DNA from selected bacteria has been isolated by Genomic Mini Kit (A&A BIOTECHNOLOGY) according to attached instructions. Isolated DNA was used as a template in PCR in which the 16S rDNA sequence was amplified. PCR conditions were as follows:

1. Preliminary denaturation step - 95ºC, 5 min.;
2. Primer annealing step - 48ºC, 1 min.;
3. Extending step - 72ºC, 1.5 min.;
4. Denaturation step - 94ºC, 1 min.;
5. Primer annealing step - 48ºC, 1 min.; and
6. Final extending step - 72ºC, 3 min.

Steps 2-4 were repeated 15 times. In PCR the S-D-Bact-0008-a-S-20 and S-*-V-Univ-1492-b-A-21 primers were used. Then the PCR amplification products were purified by GenElute™ (SIGMA) and separated by 1% agarose gel electrophoresis with ethidium bromide added to gel (0.5 µg/mL). The electrophoresis conditions were as follows: TBE buffer (1x), 60 mA, 45 min. Purified amplicons 16s rDNA were sequenced by Genomed, Warsaw, Poland. Sequencing results were analyzed by VectorNTI software (Invitrogen) and BLAST base (www.ncbi.nlm.nih.gov).

Results and Discussion

In the probes of raw materials used in the five distilleries from the Wielkopolska region, the total number of mesophilic bacteria were tested. Moreover, the number of lactic acid bacteria, anaerobic bacteria, moulds and yeasts was counted. In the A, B, and C distilleries the raw material used was corn, in D it was triticale, and in E rye. The results of this experiment are presented in Fig. 1. The level of contamination is higher in the case of the first distilleries (A, B, C) than in D and E because of the different raw materials used. The first three distilleries used corn, which might be more susceptible to contamination than triticale and rye because of the larger grain surface. In the A, B and C distilleries the total number of mesophilic bacteria, lactic acid bacteria and anaerobic bacteria was similar, but it was higher than the value presented in literature (e.g., the number of cereal grains total mesophilic bacteria is assessed at $5 \cdot 10^4$-$1.6 \cdot 10^6$ CFU/g) [14]. The level of triticale and rye microbial contamination was comparable to the value presented in literature for this kind of grain (mesophilic bacteria number for wheat reaches 10^5-10^6 CFU/g) [15]. In all five distilleries, the number of moulds and yeasts was on the lowest level. The differences between microbiological purity of raw materials among the distilleries may result not only from different grain types but also from different storage conditions. Moreover, poor quality substrates also are used for fermentation. Some grains are broken or humid, and thus more susceptible to microbial contamination.

The levels of microbial contamination of the fermentation mashes were also checked. The sweet mash, the sweet mash after 24-hour fermentation, and the mash after complete fermentation were tested. The results of these tests are presented in Fig. 2.

In Fig. 2a results from the A distillery are shown. In this distillery, the highest contamination level of total mesophilic bacteria, lactic acid bacteria and anaerobic bacteria was in the sweet mash after 24-hour fermentation. In the mash after fermentation, the highest number of anaerobic bacteria was observed and there were no moulds. Fig. 2b presents the contamination level in the B distillery. The highest level of total mesophilic bacteria and anaerobic bacteria was observed in the mash after fermentation. There were no moulds in the sweet mash after 24-hour fermentation and in the mash after complete fermentation. In Fig. 2c the microbiological situation of the C distillery is presented. After 24 hours the highest level of total mesophilic, lac-

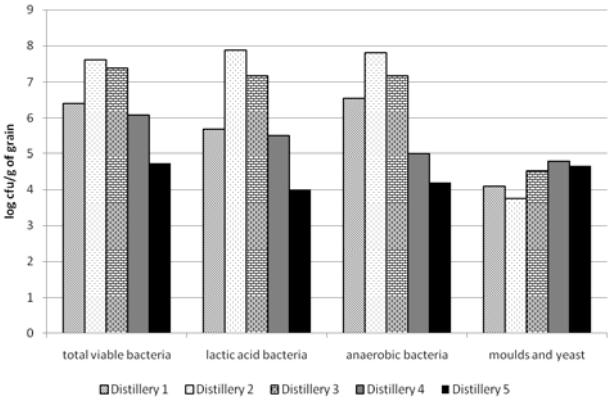

Fig. 1. Microbiological purity of raw materials from the five Wielkopolska distilleries.

tic acid and anaerobic bacteria in the sweet mash was observed. After complete fermentation the number of lactic acid bacteria decreased and the number of total mesophilic and anaerobic bacteria increased. Both in the sweet mash after 24-hours and in the mash after fermentation, there were no moulds, like in the B distillery. In the C distillery, there were fewer total mesophilic bacteria, lactic acid bacteria and anaerobic bacteria, in the sweet mash and in the sweet mash after 24-hour fermentation, than in the A distillery. In the D distillery, the level of total mesophilic bacteria in sweet mash was the lowest (Fig. 2d). In this distillery, the increase of total mesophilic, lactic acid and

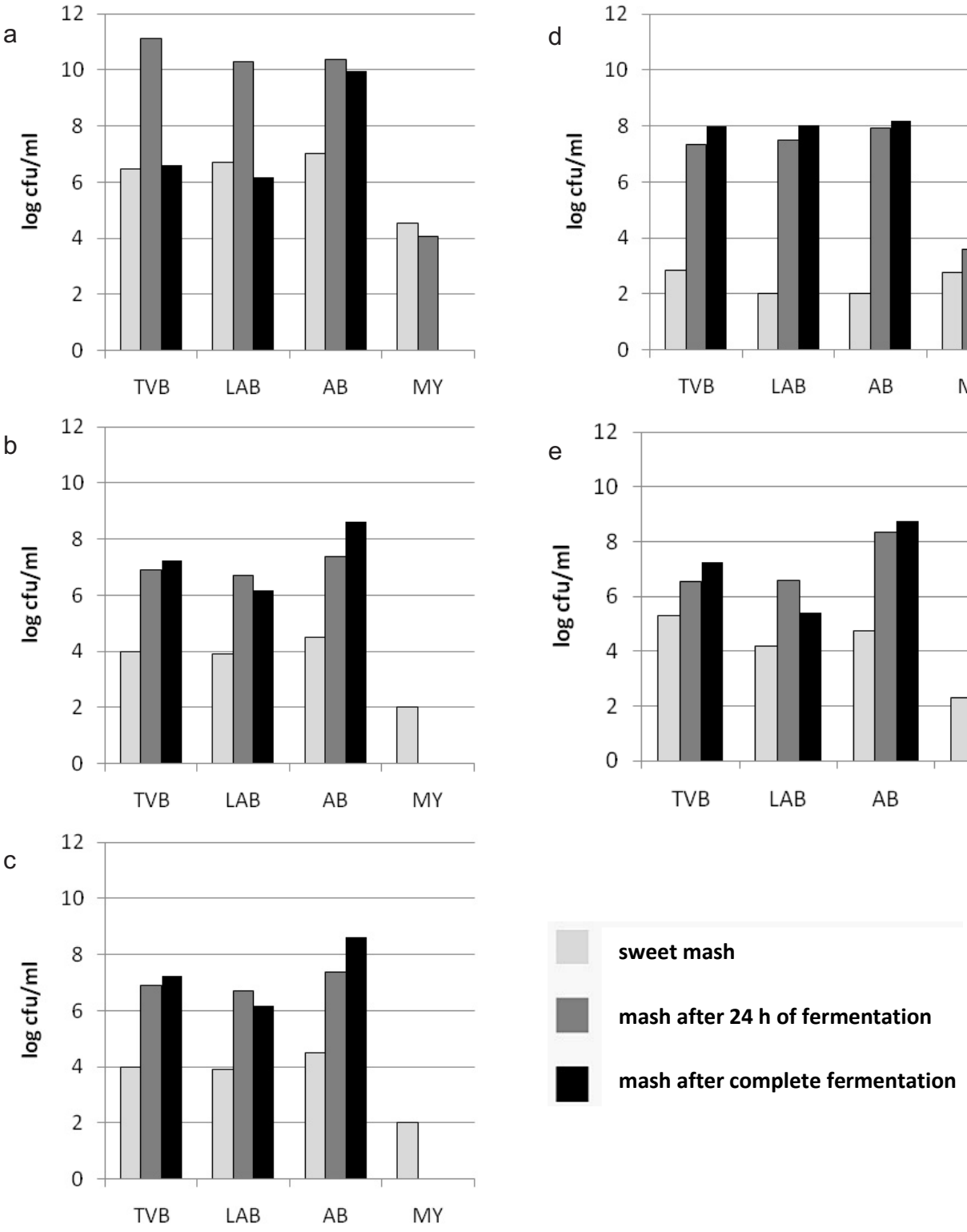

Fig. 2. The number of microorganism-contaminated grains in distilleries.
a – A distillery, b – B distillery, c – C distillery, d – D distillery, e – E distillery [log cfu/mL of mash].
TVB – total mesophilic bacteria, LAB – lactic acid bacteria, AB – anaerobic bacteria, MY – moulds.

anaerobic bacteria and moulds in the sweet mash after 24-hour fermentation was observed. The levels of these groups of microorganisms in mash after 24 hours and after complete fermentation were similar. After complete fermentation there were no moulds. Finally, in the E distillery the increased levels of total mesophilic and anaerobic bacteria were observed (Fig. 2e). In this distillery, as in the A, C and D distilleries, no moulds were observed in the mash after fermentation. An increase of total mesophilic bacteria and lactic acid bacteria in this distillery after complete fermentation was observed. However, the number of lactic acid bacteria finally decreased and after final fermentation no moulds were found. Of course fermentation is an anaerobic process and this kind of microorganism needs oxygen to survive, so they should not be observed.

To our knowledge, only a few quantitative studies on the natural occurrence of bacteria in commercial fuel ethanol plants are available. Thus, it is difficult to compare and discuss the obtained results with other results. Nevertheless, we have some evidence that bacterial contamination in commercial corn-based fuel does occur (Skinner and Leathers' experiments). The contamination level found in our study is much higher than the level in the above-mentioned work [16].

In corn grains used as a raw material for bioethanol production in the A distillery we identified the following bacteria species: *Lactobacillus* spp. (30% of all isolates): *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus lactis; Enterococcus* spp. (30% of all isolates): *Enterococcus hermanniensis, Enterococcus faecium, Enterococcus casseliflavus; Enterobacter* spp. (20%, of all isolates): *Enterobacter ludwigii, Enterobacter cloacae*; and others (20% of all isolates): *Klebsiella pneumoniae*. From the corn mash (in the same distillery) we isolated: *Lactobacillus* spp. (70% of all isolates): *Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus casei, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus lactis, Lactobacillus paracasei, Bifidobacterium* spp. (20% of all isolates): *Bifidobacterium adolescientis, Bifidobacterium angulatum*); and others (10% of all isolates): *Leuconostoc carnosum, Pediococcus parvulus*. Bacteria *Enterococcus* spp. and *Enterobacter* spp. found in the corn grains indicate the occurrence of some animals in magazines where the grains were stored (this kind of microflora is characteristic of animal alimentary tracts). All *Lactobacillus* species identified in grain were also found in sweet mash. Probably the source of these bacteria is not grain (because of the hard pre-treatment conditions), but distillery equipment. However in distilleries using modern technology without heat pre-treatment it could be a serious problem, because – as described before – *Lactobacillus* cells produce lactic acid toxic to yeasts. Thus, ethanol efficiency decreases. In corn grain we also found *Klebsiella pneumoniae*, a pathogenic bacteria that might be dangerous to the health of distillery employees. Thus, controlling the microflora is very important both for the process economy and for the health of people working in distilleries.

Conclusions

The results of our study show that distilleries in the Wielkopolska region experience numerous difficulties with microbial pollution, resulting in low ethanol efficiency. A decrease of ethanol production leads to huge economical problems in Wielkopolska's distilleries. The major contaminating microflora of the ethanol fermentation process is *Lactobacillus* spp. This kind of bacteria was also found using raw materials. This result informs us that raw material, besides fermentation equipment, water, and bioreactors, is a source of serious microbial contamination in ethanol production. Thus, control of microbial pollution is very important, and a significant reduction of microbial contamination makes possible increases in ethanol production and all the Wielkopolska distilleries would become more reliable on the Polish alcohol market.

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