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n of Bacterial Contamination in Fuel Ethanol Fermentation in Southeastern Mexico

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Abstract

Ethanol factories are susceptible to bacterial contamination, which decreases their productivity. The main studies about bacterial contamination are realized on distilleries that use corn or beet as raw material, but not molasses of cane sugar as raw. *Acetobacter* and lactic acid bacteria are the main bacterial contaminant in distilleries that use corn as raw. In this study, the contaminant bacteria in an ethanol factory which use molasses of cane sugar as raw were identified. Seven strains were isolated and belong to the genera *Acetobacter*, *Enterocccus, Klebsiella* and *Cronobacter*. All strains were able to metabolize glucose, mannitol, rhamnose, sucrose, melibiose and arabinose and were resistant to penicillin, but susceptible to enoxacin and netilmicin. This research identifies microorganisms that could be found in distilleries that use molasses as raw in tropical countries.

Keywords: Ethanol factory; Bacteria; Carbohydrates

Abbreviations

MRS: Man, Rogosa and Sharpe; EMB: Eosin Methylene Blue; WLD: Wallerste in Differential; PBS: Phosphate Buffered Saline; PCR: Polymerase Chain Reaction; ONPG: 2-Nitrophenyl-ß- D-Galactopyranoside, ADH: L-Arginine; LCD: L-lysine; ODC: L-ornithine, CIT: Trisodium Citrate; H2S: Sodium Thiosulphate; URE: Urea; TDA: L-tryptophane; VP: Sodium Pyruvate; GEL: Gelatin; GLU: D-Glucose; MAN:D-Mannitol; INO: Inositol; SOR: D-Sorbitol; RHA: L-Rhamnose; SAC: D-Sucrose; MEL: D-Melibiose; AMY: Amyddalin; ARA: L-arabinose; LAC: D-Lactose; MAL: D-Maltose; SAL: Salicin; XYL: D-Xylose; ESC: Esculin; GLY: Glycerol; CEL: D-Cellobiose; MNE: D-Mannose; MLZ: D-Melezitose; RAF: D-Raffinose; THE: D-Trehalose; AK: Amikacine; AM: Ampicillin; CF: Cephalothin; CRO: Ceftriaxone; CL: Chloramphenicol; DC: Dicloxacillin; ENX: Enoxacin; ER: Erythromycin; GE: Gentamicin; NET: Netilmicine; PE: Penicillin; SXT: Trimethoprim-Sulfamethoxazole; CFU: Colony- Forming Unit; S: Susceptible; R: Resistant; I: Intermediate; A10-CAD; B2-CAD; D-CAD; E2-CAD; 7-CAD; 8-CAD: isolated strains

Introduction

Ethanol can serve as an alternative biofuel. It is produced during the fermentation of easily of low cost substrates, such as: corn, sugar cane juice, molasses cane juice, molasses beet juice, cassava, potato, hemicellulose substrates (paper sheet, sawdust) [1]. USA and Brazil are the main ethanol producers at worldwide, corn and sugar cane molasses are used as raw materials, respectively [2].

Ethanol fermentation is carried out by yeast; cell viability of yeast is adversely affected by the acid organics produced by bacteria contaminant (i.e. acetic and lactic acid). Mexico as Latin America use sugar cane and molasses cane juice as biomass for ethanol production, due to its great abundance, easy culture and fermentation [3]. Distilleries in Mexico are located mainly in the southeast where the weather is tropical (40-45°C).

In distilleries, the media is not sterilized and only diluted molasses are used (near 22°Brix). The main contaminants in the fermentation tanks that use corn as raw material are Lactic Acid Bacteria (LAB) mainly *Lactobacillus* [4]. Although others LAB have been found such as: *Leuconostoc, Bifidobacterium, Lactococcus, Pediococcus* [5-10]. *Acetobacter* and *Weisella* strains have been found also in distilleries; *Acetobacter* utilize simple carbohydrates and ethanol as carbon source to produce acetic acid [11,12]. These genera affect the process because consume the carbon source, hence, the yield and productivity decrease [7,10,12,13,14]. Besides, organics acid produced by bacteria contaminant as acetic and lactic acid, adversely affect cell viability of yeast [4,15].

To reduce the microbiota contaminant in fermentation broth antimicrobial agents are used. Penicillin, tetracycline, monensin, virginiamycin, polymyxin B [16], hydrogen peroxide, potassium metabisulfite [17], tartaric acid [4] are the most common. Generally, the factory does not make a study to determine which antimicrobial agent could eliminate the contaminant microbiota. This general approach often results in the generation of antibiotic resistant bacteria, making the antibiotics less efficient in the reduction of contaminants. In this paper a microbial community representative of the fermentation and storage tanks of an ethanol factory was studied biochemically and molecularly in order to identifying which antibiotics could remove it. Therefore, this study is relevant for distilleries in tropical countries which produce ethanol via fermentation of molasses from cane juice.

Materials and Methods

Sample collection and bacteria isolation

Samples were collected from fermentation broth every 4 hrs, since pre-fermentation step until fermentation end in ethanol factory. At the molasses storage tank, the samples (500 g) were collected from different sites and homogenized. After, samples were diluted in decimal dilutions in PBS buffer and 100 µL were plated by duplicate on several selective agar mediums: MRS-Itraconazole (Fluka, Germany), WLD (Fluka, Germany) and EMB (Dibico, Mexico). Petri dishes were transported to Molecular Biology Laboratory of the Universidad del Papaloapan and were incubated at 37°C for 48 hrs in CO2 incubator (anaerobic conditions). Colonies with morphologic differences were selected and isolated.

Molecular and biochemical identification

Colonies were characterized at the morphologic level, with biochemical (API 20A and 20E, Biomerieux, France) and molecular tests, Gram's Method (Hycel, México). To molecular characterization, genomic DNA was extracted using the Ultra Clean microbial DNA isolation kit (MoBIO, USA). The 16S rDNA was amplified with the primers fD1 (CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG) and rD1 (CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC) by [18]. PCR products were purified, sequenced (Macrogen, Korea) and analyzed through the maximum likelihood method, with Nei Tamura model, and 1500 bootstrap replicates [19].

Antibiotic sensibility testing

Sensitivity testing was performed to the CAT manual (BIORAD), which contains: AK (30 µg), AM (10 µg), CF (30 µg), CRO (30 µg), CL (30 µg), DC (1 µg), ENX (10 µg), ER (15 µg), GE (10 µg), NET (30 µg), 10 U PE and SXT (25 µg). Strains were grown according to supplier recommendations.

Results and Discussion

Microbiota description

Bacteria, yeast and molds were obtained in all growth media, but only bacteria were characterized. Bacteria from the fermentation tank grew mainly on WLD medium, some strains produced turn on agar color, from blue to yellow, due to the acidification of the medium. Yellow area around colony gives an indication of the amount of lactic or acetic acid produced by the colony. On EMB medium grew native yeasts and LAB from molasses on MRS-Itraconazole medium were isolated.

Figure 1 summarizes the bacterial behavior during pre (0-3 hrs) and fermentation steps (4-28 hrs). Seven strains with different macro and microscopic characteristics were isolated; the largest microbial count was detected at the end of the prefermentation step and in the beginning of fermentation step. At the begin of fermentation step (0 hrs), fermentation broth has around 22° Brix and the fermentation end (24 hrs) has 10° Brix. B2-CAD showed the largest bacterial count for the bioprocess and its concentration was increased in both steps. Unlike A-CAD, B-CAD, C-CAD and E-CAD only were observed during prefermentation step. Instead, D-CAD was observed at the end of the prefermentation and in half of the fermentation step. On the other hand, E2-CAD was only observed at the end of the fermentation. Therefore, B2-CAD can grow as the fermentative yeast of the bioprocess, instead, E2-CAD grow better at fermentation end, when sugar concentration is low. The total microbial concentration was around 3000 CFU/ml, lower than the quantity reported of microbial contaminant from sugar cane juice, where the microbial concentration was approximate 105-108 CFU/ml [4,20]. The low native bacterial concentration observed in this work is an advantage to produce ethanol from molasses and avoid economic lost by contaminant bacteria. B-CAD and C-CAD strains are natives yeast and E-CAD strain is a mold; these isolated were not characterized. A-CAD and E2-CAD were grouped in the clade of *Enterococcus*, B2-CAD was added in *Acetobacter* cluster and D-CAD in *Cronobacter* clade (Figure 2, Table 1). From molasses yeast (9-CAD), bacteria (7-CAD and 8-CAD) and mold (10-CAD) were detected. Bacteria were isolated in MRS-Itraconazole medium and the yeasts and mold of EMB medium. Microbial concentration for each isolated oscillated between 60-560 CFU/ml (Table 2). The bacterial concentration was lowest than fermentation tank, probably by the high concentration of carbohydrates in molasses (around 80° Brix). This shows that the contamination is largest during the fermentation step and the distilleries must focus in this phase. 7-CAD was clustered with the *Klebsiella* genus and the 8-CAD was not associated with a particular clade (Figure 2, Table 1). Even though *Klebsiella* has not been found as contaminant typical in distillery factories, it has been found frequently associated with sugar cane as growth factor [21]. Therefore, it could come from there.

Figure 1: Kinetics of growth of bacteria, yeast and fungi during fermentation process.

Figure 2: Phylogenetic tree of microbiota isolated from fuel factory.

Table 1: Information general of the local alignment of the sequences obtained of the isolated.