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Identification and biological control of microbial agents causing corruption of stored sugar beets in sugar production industry

S. Noori[(1)], N.S. Naghavi[(2)]*, M. Mohammadi Sichani[(3)], M. Gol Gol Jam[(4)] and M.A. Zia[(5)]

[(1)] *Msc student of microbiology, Islamic Azad University of Falavarjan, Falavarjan, Iran.*
[(2)] *Assistant professor of microbiology, Islamic Azad University of Falavarjan, Falavarjan, Iran.*
[(3)] *Instructor of microbiology, Islamic Azad University of Falavarjan, Falavarjan, Iran.*
[(4)] *Expert of microbiology, Islamic Azad University of Falavarjan, Falavarjan, Iran.*
[(5)] *Assistant professor of Islamic Azad University of Khorasghan, Khorasghan, Iran.*

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ABSTRACT

There is a long period distance between sugar beet harvest and its transport to sugar factory. Sugar beet crop is damaged and injured during harvest and transport which provides a suitable place for various saccharolytic microorganism growths in terms of temperature, moisture, pH and glucose concentration. In this study, samples were randomly selected from root stored in spring 2010 and also the processed syrup in one of the sugar factories in Isfahan, in order to isolate and identify microorganisms. After washing and disinfecting root surface, infected pieces were taken from different regions of the tissue, were washed, and microorganisms were isolated and identified using standard microbiological methods. Different types of gram-positive *bacilli* and *cocci*, including *Bacillus*, *Leuconostoc*, *Staphylococcus*, and *Streptococcus* from bacteria, and several types of fungi including *Paecilomyces, Chrysosporium*, *Pencillium*, *Fusarium* and *Pythium* were isolated and were purified. Pathogenic bacteria such as *Staphylococcus aureus* and *Staphylococcus saprophyticus* were detected among isolated bacteria. Bacterial counting in syrup collected after heat processing showed the presence of bacterial spores and the growth of bacteria as 53 colony forming unit per milliliter after vegetation. The bacterial population which remained in processed syrup was *Bacillus* species. These bacteria are called as 'opportunistic pathogens' and some of them produce allergens. Therefore, it is important to control their population in stored sugar beet which can affect the quality of health parameters. Ethanol extract from bee Propolis had significant effect on isolated microorganisms. The minimum lethal concentration of Propolis was 6 times less than sodium hypochlorite and 12 times less than calcium hypochlorite.

Keywords: Bee Propolis, calcium hypochlorite, microbial counting, sodium hypochlorite, sugar beet, storing

INTRODUCTION

Owing to the root damage and high carbohydrate contain, sugar beet is a proper place for microorganism accumulation which resulted in sucrose decomposition and yield reduction. In addition to damage caused by microorganism activity, organic acids such as lactic acid and polysaccharid compounds are produced and nitrate is also converted to nitrite. These phenomenons not only cause a sharp decrease in sugar content and increase in microbial infection of the syrup but also may cause several problems in sugar production process. If the microbial activity is not controlled, in addition to above problems, it will cause final product loss and industrial pollution such as syrup viscosity increase owing to sucrose conversion to dextran. This conversion may clog syrup transfer path, cause reduction in heat transfer, evaporation efficiency, and crystallization efficiency, crystals deformation, centrifuge clog, and sucrose loss in molasses. Crude syrup derived from degrading sugar beet includes extra acids (due to microorganism activity) which require more lime to neutralize it (Belamri *et al*. 1991). In a study by Belamri *et al*. (1991) in sugar factories in Morocco, bacteria *bacillus* species were found

*Corresponding author's email: naghavi@iaufala.ac.ir

as main pollutant of crude syrup. Sucrose decomposing bacteria were isolated from crude syrup and different *bacillus* species including *B. stearothermophilus, B. subtilis, B. pumilus,* and *B. psychrosaccharolyticus*. Dakkari *et al*. (1992) studied the industrial value of sugar beet in Mediterranean climate. Microbial testing of dry matter and juice of both normal and degraded sugar beet samples illustrated higher microbial counts and lower quality. To solve this problem, increasing storage quality is emphasized. Alimoradi (2010) studied the impact of post-harvest loss in silo and its association with root fungi in sugar beet. His results showed that after a long time, for example, 120 days of sugar beet storage, pollutants had adverse effect on extractable sugar content. However, it is difficult to accurately predict loss rate before harvest. Based on these results, it is recommended that harvest should not be taken from infected fields, and if the harvest was done, sugar beet samples should be consumed before storage. Most of the studies which were taken on plant pathogenic factors were related to identification and control of both grain and crop spoilage during cultivation. However, one of the main problems in processing industry is the crop spoilage and contamination after harvest until its conversion to the desired product. Since crop harvest is performed in a certain time period, crop storage is inevitable. Propolis is a resin material (the main ingredient is resin and wax) collected by bees and is well-known for its antimicrobial activity (Greenaway *et al*. 1990; Bahrami *et al*. 2009). Different studies indicated microorganism control by different propolis extract concentration (Uzel *et al*. 2005). Propolis has strong bacteriostatic property (Rahman *et al*. 2010) which is used in drug industry activities such as anesthetic, desensitizing, etc. (Uzel *et al*. 2005, Bufalo *et al*. 2009, Fuliang *et al*. 2005). In this study, factors causing sugar beet spoilage in silo were identified and were isolated. Also, the bacterial contamination rate in the processed syrup was evaluated by counting bacteria and their spore as an index of microbial contamination (remaining after thermal process). Remaining bacterial population after syrup preparation was identified. The antimicrobial effect of propolis, as a biological control agent, on the isolated microorganisms was evaluated in comparison with two common disinfectants (sodium hypochlorite and calcium hypochlorite).

MATERIALS AND METHODS

Sampling

Samples were taken from roots collected in spring 2011 and stored in Nagsh Jahan sugar factory. 10 samples were collected in two phases from three silos with 30 tons capacity. Random sampling was done from rotten roots in order to isolate and identify microorganisms.

Isolation and cultivation of bacteria

Based on Leuven and Croylaan (2000) method, after washing and disinfecting with 70% ethanol, infected regions were isolated and were smashed. Then, the extraction was mixed with distilled water and different concentrations were prepared. Bacteria were transferred to Trypticase Soy Broth (TBS) media and after bacteria enrichment in this environment, isolation and purification were carried out on Nutrient Agar (NA) at room temperature and pH=7. Bacteria were detected in sugar beet and syrup samples using standard microbiologic methods through staining and biochemical tests.

Total bacteria count in syrup

Fresh samples were taken from syrup before crystallization step. After dilution with sterile distilled water, 1 ml of each diluted sample was added to NA medium and was incubated at 37 ^{o}C for 24 h. Spore growth rate and vegetative forms were reported based on colony forming units per ml (CFU.ml^{-1}) (Mohan *et al*. 2010).

Bacteria's spore counting in syrup

To count spore number in syrup, vegetative forms were destroyed in water bath at 80 ^{o}C for 10 min and spores were left alone. Different dilutions were prepared and one ml of each was added to NA medium. The cultures were incubated at 37 ^{o}C for 24 h. Counting was done based on CFU.ml^{-1} (Mohan *et al*. 2010).

Fungi isolation and cultivation

Small parts of the infected tissues were taken from various sample parts and were placed in PDA (Potato Dextrose Agar) and SDA (Doboraud Dextrose Agar) media at room temperature and pH=6 (to prevent bacteria growth). After several culture replications, fungi and yeasts were purified and were recognized using microscope, culture slide, and identification keys (Samson *et al*. 2004).

Table 1. Isolated gram-positive *coccus* properties

Bacteria	Fermentation on manitol salt agar	Growth on Bile esculin agar	Antibiotic sensitivity	Sucrose fermentation	Catalase	Coagulase	Hemolysis	Microscopic morphology
Staphylococcus aureus	+	-	Susceptible to neobacin	+	+	+	Gamma	Cluster
Staphylococcus saprophyticus	+	-	Resistant to neobacin	+	+	-	Gamma	Cluster
Staphylococcus epidermidis	-	-	Susceptible to neobacin	+	+	_	Gamma	Cluster
Leuconostoc mesentroides	+	+	Resistant to vancomycin	+	-	-	Gamma	Cluster & binomial
Streptococcus Spp.	-	variable	–	+	-	-	Beta	Chain

Table 2. Isolated gram-positive *bacillus* spore properties

Bacteria species	Lecithinase	Lipase	Mannitol Lipase fermentation	Xylose fermentation	Sorbitol fermentation	Sucrose fermentation	movement	MR	VP	Coagulase	Hemolysis
Bacillus bervis	+	+	+	+	+	+	+	+	-	+	Beta
Bacillus pumilus	-	+	Variable	Variable	Variable	+	-	+	+	-	Beta
Bacillus stearotermophilus	-	+	+	+	Variable	+	+	-	+	-	Beta
Bacillus subtilis	-	-	+	Variable	-	+	+	+	+	-	Beta

Slide culture method

This method is used for mold fungi identification. In this method, reproductive organs of fungus are characterized in three-dimensional format and morphological characters can be detected. First, a piece of SDA medium (1 cm^2) was placed on glass slide center. Then glass slide was placed horizontally on U-shaped tube into a large glass plate. Using sterile ounces, the fungus was cultured on four points of the slide and a disinfected coverslip was placed on agar. To prevent agar drying during incubation period, approximately 10 ml sterile distilled water was added into plate and the lid was put. Plates were kept at room temperature for one week. After incubation, coverslip was removed. Two drops of lactophenol cotton blue dye were poured on a clean glass slide and the coverslip was placed (Leuven Croylaan 2000). Thallus structure was studied by light microscope.

Propolis ethanol extract preparation

25 g propilis was dissolved in 500 ml 96% ethanol and the solvent was filtered through whatman filter paper. Impurities obtained by filter were weighed and differed from solvent dry matter.

Effect of antimicrobial agents on bacteria and isolated fungi

The extract obtained from 96 well microplate was diluted. 5% dimethyl sulfoxide was used as solvent and sodium hypochlorite and calcium hypochlorite (as chemical disinfectants) were diluted with distilled water in a 96 well microplate. 100 µl of each dilution was poured into each well and 100 µl of microorganism equal to 0.5 McFarland standard growth was added. A control well containing infected microorganism and another well containing solvent were also included. A solution of antimicrobial that prevents microorganism growth was considered as the Minimum Inhibitory Concentration (MIC). To estimate the Minimum Bacterial Concentration (MBC) and the Minimum Fungicidal Concentration (MFC), microorganisms were transferred from well containing MIC and two wells before it, into NA (for bacteria) and PDA (for fungi) media (Rahman *et al*. 2010; James 1998). Data from three replications were analyzed using Minitab software and t-test was used for mean comparison.

RESULTS

Isolation and identification of bacteria

Isolated bacteria were from gram-positive *bacillus* and *coccus*.

Total bacteria and spore counting in sugar beet syrup

A few percent of the spores remained in the processed syrup before crystallization. Number of colony forming unit (CFU) for all bacteria and spores was 53 $CFU.ml^{-1}$ in which 3 $CFU.ml^{-1}$ was spore composition (5.9%). Significant difference was found between bacteria number before thermal processing and remained spore number after processing ($P < 0.05$). However, *bacillus* bacteria were remained in syrup after processing stage.

Table 3. Mean comparison of the bacteria number before syrup processing and remained spore number after processing

Tasting phase	Number of bacteria per ml (CFU.ml^{-1})	t	Probability level
Before heat processing	53	28.57	0.043
After heat processing	3		

Fungi isolation and identification

Fig. 1-5 show macroscopic and microscopic morphology of isolated and cultured fungi using slide culture method. Isolated fungi were belonged to *paecilomyces*, *chrysosporium, penicillium, fusarium*, and *pythium* species.

Effects of propolis ethanol extract on sugar beet isolated bacteria

Fig. 6 shows the effects of different propolis ethanol extract on isolated bacteria.

Resistant bacteria species including *staphyloccus aureus*, *asylum broyes*, *bacillus stearothermophilus* had MIC in 0.1 mg/ml and MBC in 0.2 mg/ml propolis ethanol extract. *Bacillus pomilus* and *leuconostoc mesenteroides* had same MIC and MBC in 0.1 mg/ml. The most susceptible bacteria were *streptococcus* species with MIC in 0.025 mg/ml and MBC in 0.05 ml/ml.

Propolis effects on isolated fungi

Fig. 7 shows the effect of different propolis concentration on isolated fungi.

Resistant isolated fungi species such as *penicillium* and *Fusarium* had MIC in 0.2 mg/ml and MFC in 0.4 mg/ml. *Pythium* had same MIC and MFC in 0.4 mg/ml and susceptible species such as *paecilomyces* and *chrysosporium* had MIC and MFC in 0.1 and 0.2 mg/ml, respectively.

Comparison of the propolis effect and two chemical disinfections

Propolis is more pronounced against bacteria and fungi than hypochlorite and calcium hypochlorite (Table 4). Table 4 indicates the minimum concentration required for bacteria and fungi control.

DISCUSSION

Belamri *et al*. (1991) isolated sucrose decomposing bacteria from sugar beet syrup and identified different *bacillus* species including *B. stearothermophilus, B. subtilis, B. pumilus,* and *B. psychrosaccharolyticus*. *Bacillus stearothermophilus, B. pumilus,* and *B. broyes* were also identified in sugar beets stored in silo which indicates that sugar beet infection in silo

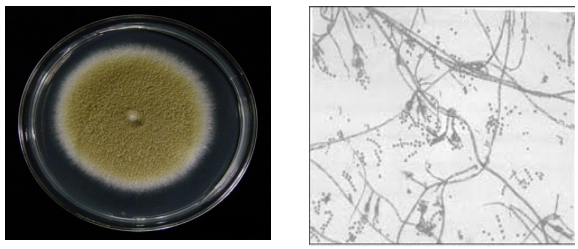

Fig. 1. Macroscopic and microscopic morphology of isolated *paecilomyces* fungi

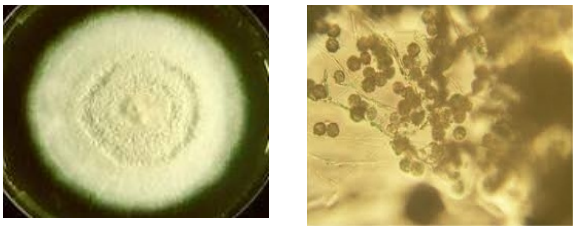

Fig. 2. Macroscopic and microscopic morphology of isolated *chrysosporium* fungi

Fig. 3. Macroscopic and microscopic morphology of isolated *penicillium* fungi

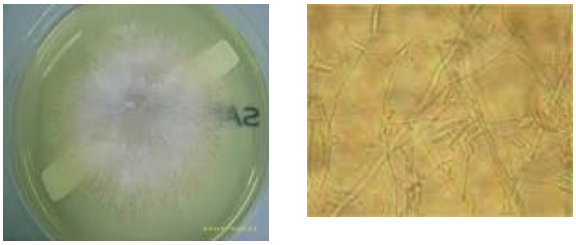

Fig. 4. Macroscopic and microscopic morphology of isolated *Fusarium* fungi

Fig. 5. Macroscopic and microscopic morphology of isolated *Pythium* fungi

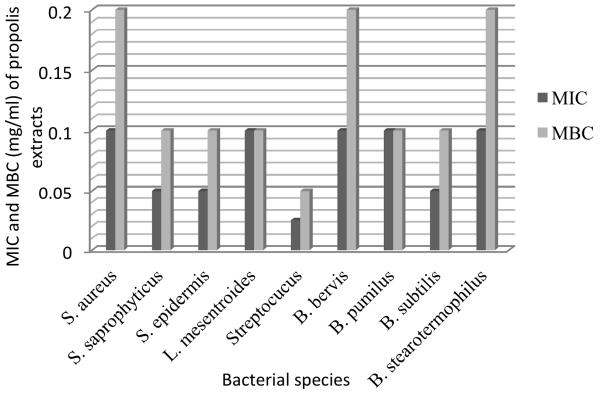

Fig. 6. Effects of different proplois ethanol extract on sugar beet isolated bacteria

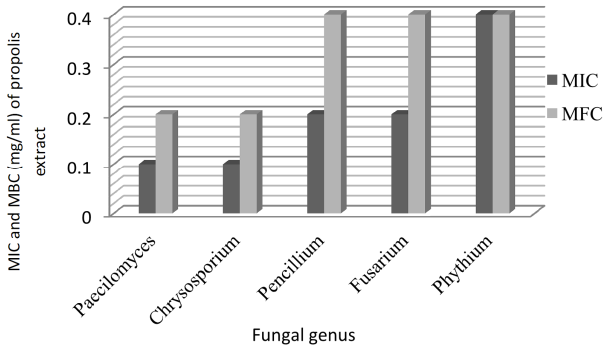

Fig. 7. Effects of different propolis ethanol extract on isolated fungi

Table 4. Statistical comparison of average MBC concentration

Control factor	MBC (mg/ml)	t	Probability level
Propolis	0.4	0.78	0.043
Sodium hypochlorite	2.4		
Propolis	0.4	1.71	0.015
Calcium hypochlorite	5		

will also cause syrup microbial infection. In a study by Alimoradi (2010), an association was observed between sugar beet loss in silo and increase in root rot by fungi origin. In this study, we also isolated *pythium* and *fusarium* fungi from stored sugar beet which assumed to be transferred from contaminated field or warehouse. Therefore, it is recommended to refuse harvesting from contaminated field and if the harvest was done, samples should be used before storage. Silo should be cleaned after storage completion and being disinfected. Lewis and Papavizas (2000) and Bardin *et al*. (2004) have emphasized that biocontrol methods can control contamination factors in plants. In this way, using fungi and yeast that have antagonist reaction to other microorganisms is recommended. Too many studies were carried out on disease occurrence in seeds stored in silo. Bardin (2004) confirmed the effects of other biocontrol methods such as using coriander straw, beans, cotton, and lentils on *pythium* fungi disease. In recent years, several studies were carried out on sugar beet pathogenic microorganism and their control during cultivation. For example, Ashraf Mansoori *et al*. (2010) introduced resistant sugar beet hybrid to plant twisting viral disease. Rouzbeh *et al*. (2011) used nano silver particles to control sugar beet contamination in tissue culture. Although sugar beet contamination in silo is one of the main problems of sugar industry in Iran, no comprehensive study was done in the field of contamination rate and microorganism type. Therefore, in this study we began with reviewing microorganism contamination and introducing a method to control them. Microbial agents isolated in this study were aerobic or anaerobic microorganisms which can break down sucrose. Thus, if any of these identified species be located in ideal condition in terms of temperature (for fungi, between 25 to 28 ^{o}C and for bacteria, between 37 to 40 ^{o}C), pH (slightly acidic and neutral bacteria), and sucrose concentration, it could have the maximum growth in a short time. Some bacterial species such as *B. broyes*, *B. pumilus*, *B. stearothermophilus,* and clostridium *thermosaccharolyticum* (sporiferius) are able to withstand 65-70 ^{o}C and pH 5-7, and isolated fungi can also tolerate temperatures less than 20 ^{o}C (shah 1999). Among isolated bacteria, pathogenic types such as *S. aureus* and *S. saprophyticus* and opportunistic pathogen such as *S. epidermis* were observed (Brooks *et al*. 2010). It is needless to say that sugar syrup is exposed to 70-80 ^{o}C at the time of preparation which removes bacterial vegetative forms. The interesting point in bacteria number counting in the processed syrup is the minimum number of spores compared with vegetative forms. This difference may show the presence of proper condition for bacterial growth in syrup after cooling that caused bacteria to complete spore stage and also their active growth. The remaining bacteria in syrup after heating were belonged to *bacillus* species. Some species such *B. pumilus*, not only decreased stored sugar beet quality but also caused illness in human and plants (Galal *et al*. 2006; Tena *et al*. 2007). Laboratory studies showed that *B. subtilis* can cause hypersensitivity and allergic reactions in body (Kawabatai *et al*. 1996). Sodium hypochlorite and calcium hypochlorite are disinfectants which have good inhibitory and cytotoxic effect on gram-positive and

gram-negative bacteria and fungi (Tully 1914; Memarian *et al*. 2005; Ebadian *et al*. 2007). In the present study, 2.4 mg/ml sodium hypochlorite, 5 mg/ml calcium hypochlorite, and 0.4 mg/ml propolis destroyed all fungi and bacteria. Thus, the antimicrobial activity of propolis ethanol extract on contamination factors was 6 and 12 times more than sodium hypochlorite and calcium hypochlorite, respectively. Compared with previous results, propolis ethanol extract had stronger antimicrobial effect than bee propolis. Rahman *et al*. (2010) studied the antibacterial effect of propolis on *S. aureus* and *Escherichia coli* and 3.5 mg/ml concentration was found to be as the best concentration. 0.2 mg/ml propolis was selected as the best concentration for *S. aureus* control. In recent years, particular attention has been paid to biological control of sugar beet disease. For example, in Austrian sugar industry, humulus lupulus, turpentine, and oil palm extracts are used instead of chemical disinfectants (Sheikh Al elesami 2010). In this study, the ethanol extract of bee propolis is recommended. This material is also used orally for disease treatment and its usage is affordable. It is recommended to be used as spraying or by immersing before storage.

CONCLUSION

In this study, different gram-positive bacteria such as *cuccuss* and *bacillus*, and fungi were isolated from stored sugar beets. These microorganisms were also found in active form after syrup processing at 70-80 ^{o}C which affects sugar production in terms of industry and health. Current sugar beet storage condition in sugar factories provides a proper situation for microbial contamination. Among identified species, *S. aureus* variety was also detected. However, after heating and syrup preparation, only sporiferius type belonged to *bacillus* species remained. Some remained bacteria were opportunistic pathogen. These spores can grow after syrup cooling and cause microbial contamination in final product. In this study, a biologic compound called bee propolis is recommended for sugar beet contamination control in silo which had strong activity in lower concentrations compared with sodium hypochlorite and calcium hypochlorite.

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