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Microbial Contamination Reduction of Sorghum (*Sorghum bicolor* L.) Stalk Juice as Bioethanol Raw Material through Microfiltration Process

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Abstract: Microbial contamination reduction for bioethanol raw material is commonly achieved through heat sterilization process. However, heat sterilization process could contribute up to 64.5% of the total energy requirement of bioethanol production; therefore microbial contamination reduction process at lower temperature is preferable. This research studied the implementation of microfiltration to reduce microbial contamination of sorghum stalk juice as bioethanol raw material, in comparison to pasteurization. The raw sorghum (*Sorghum bicolor* L.) stalk juice contains microbial contaminant load in the form of bacteria, yeast and spore-forming mold. Effectiveness of microfiltration was observed by processing sorghum stalk juice through micro filter with nominal pore size of 3.0 μm, 1.0 μm, and series of 3.0 μm and 1.0 μm. Pasteurization was conducted at 70^{o}C for 30 minutes in an autoclave. Total plate count analysis showed that pasteurization reduced total microbial load to 99.93% while microfiltration using pore size of 3.0 μm, 1.0 μm, series of 3.0 μm and 1.0 μm showed non-significant difference with total microbial load reduction of 99.99% In 7 days of storage in room temperature, juice treated by microfiltration using pore size 3.0 μm maintained its total microbial load of 1.12 ± 0.01x 10^6 CFU/ml, juice treated with microfiltration of pore size 1.0 μm has 11.60 ± 0.74x 10^3 CFU/ml, series of filter gives 8.50 ± 0.64 x 10^3 CFU/ml, while microbial load of pasteurized sorghum stalk juice increase to 1.06 ± 0.02 x 10^9 CFU/ml. Suitability of process as pretreatment for bioethanol raw material was observed through inoculation of *Saccharomyces cerevisiae* in the treated juice. Yeast growth in filtered media gives Yx/s of 0.49 g biomass/ g substrate, higher than pasteurized media (0.44 g biomass/ g substrate). It could be concluded that microfiltration using nominal pore size of 1.0 μm is sufficient to reduce microbial contamination for sorghum juice as bioethanol raw material.

Keywords: Microfiltration, microbial contaminant, sorghum stalk juice, bioethanol

1. Introduction

Sterilization is required to ensure that microbial contamination does not interfere with fermentation process. In various processes, including the fermentation of bioethanol, sterile condition is achieved through heat process using direct or non-direct contact saturated steam (Siriyotha *et al*. 2006). Sterilization is also required for fermentation of bioethanol using sorghum stalk juice as raw material. Juice extracted from sorghum (*Sorghum bicolor* L.) stalk is containing various type of naturally occurring microorganisms, including spore forming molds, yeasts, and bacteria (Kundiyana *et al*. 2010). Sorghum stalk juice is also having sugar content of 7-14% which makes it suitable growth media for microorganism, although it is known that the stalk may contain high concentration of phenolic compound (Salmon 2006; Towo *et al*. 2006). Previous study showed that in clarified sorghum stalk

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juice using milk of lime that includes heating the juice to 80^{o}C, microbial contamination is still observable (Kartawiria *et al*. 2015).

Previous studies also indicated that sterilization involving high heat in bioethanol raw material preparation negatively contributes to the net energy requirement of overall process. Heat requirement in the form of saturated steam contributes up to 50.7 – 64.5% of overall energy requirement for producing bioethanol(Lavert-Ofir *et al*. 2008; Lorenz & Morris 1995; Pimentel *et al*. 2007).Steam is mostly involves in material hydrolysis, sterilization, and separation. Bioethanol separation and purification requires the highest energy compared to other steps of process (Scheller and Mohr, 1972; Schmer *et al*. 2008). Study on low energy microbial reduction has been conducted using pasteurization and filtration. Pasteurization of sorghum stalk juice at 70-75^{o}C for 30 minutes reduces microbial load and increases juice shelf life significantly (Kumar *et al*. 2013; Mazumdar *et al*. 2012). Filtration could be conducted using various filter size. Microfiltration (0.1-10 μm) and ultrafiltration (0.01-1.0 μm) is interesting for application since most bacteria has size between 0.2 to 5 μm (Stanbury & Whitaker 1984). Microfiltration, specifically has been studies for handling of liquid foodstuff and fermentation broth (De-Carvalho & Da-Silva 2010; Zabed *et al*. 2014). Study on sugar cane juice shows that microfiltration is effective for clarification and color removal (Suprihatin 2007). The application for sorghum stalk juice however needs to be studied to evaluate its suitability. This research studied the implementation of microfiltration to reduce microbial contamination of sorghum stalk juice as bioethanol raw material, in comparison to pasteurization.

2. Materials and Methods

2.1. Clarification of Juice

Sorghum stalk juice was extracted from red sorghum stalk 5-6 month old, filtered and clarified. Clarification was using milk of lime (MOL) of 300 ml for 1 liter of juice. Liming were conducted at 80^{o}C for 30 min, then settled and decanted to obtain the clear juice. Juice pH was buffered to reach 6.5. Phenolic content were reduced by running the juice into activated carbon column with retention time of 120 min.

2.2. Pasteurization and Filtration

Pasteurization were conducted by heating 200 ml of sorghum juice in autoclave (Hirayama, Japan) at 70^{o}C for 30 min. Filtration were conducted through 3 treatments, namely Filter A with pore size of 3.0 μm (DEWater DW003SE, China), Filter B (1.0 μm, DEWater DW001SE) and Filter C (series of filter 3.0 μm and 1.0 μm). The juice was pumped through the filter using RO pump (Deng Yuan TYP2500N, China) at maximum pressure of 80 psi and flow rate of 0.6 l/min. All filtration lines were sanitized using 70% ethanol solution. Filtered juice contained in sterile Erlenmeyer flasks for 7 days at 25^{o}C.

Microbial load were measured using Total Plate Count Method on TPC agar (Merck, Germany). Incubation was conducted at 37^{o}C for 24 hour. TPC were measured for 7 days of storage. Manual tally and LabMate (SIIS) software were used for colony counting.

2.3. Microbial Growth

Pasteurized and filtered sorghum stalk juice were inoculated with *Saccharomyces cerevisiae* ATCC18824/ BTCC Y-34, to evaluate the suitability for bioethanol production. 200 ml of treated sorghum stalk juice were inoculated using 20 ml inoculum aseptically in aerobic condition in stirred Erlenmeyer flasks. Inoculum was prepared using Nutrient Broth (Merck, Germany) enriched with 10% of glucose as carbon source. Inoculum was kept at logarithmic phase. The aeration was provided from filtered air pump at the rate of 0.8 VVM. Growth kinetics was followed by turbidometry method. Turbidity of samples (2 ml) was measured by Spectrophotometer (GeneSys, UK) at 600 nm wavelength. Sugar concentration was evaluated using DNS method.

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3. Results and Discussion

3.1. Microbial Removal

Pasteurization and microfiltration could reduce the microbial load in sorghum stalk juice significantly. Microorganism concentration in raw sorghum juice after pressing was 2.23×10^9 CFU/ml. Pasteurization could reduce microbial concentration up to 99.93%. Effectiveness of pasteurization is slightly higher in comparison to clarification process, which also involving heating the juice. Sorghum juice used as control was the clarified juice with microbial concentration of 2.91×10^6 CFU/ml. Microfiltration using Filter A, B and C did not gives significant differences, at approximately 99.99% reduction. Sterile condition, as expected, is not reached through microfiltration.

During 7 days of storage, pasteurized juice maintained low microbial load up to day 2, then TPC start to increase. Previous study mentioned that microorganisms on the surface of sorghum stalk are consisting of bacteria, yeast, and spore forming molds (Boone *et al*. 2013; Gassem 1999). Pasteurization could kill most of the microorganism; however spores started to grow after incubation at appropriate temperature. At 7 days of storage, the microbial growth is as presented in Figure 1.

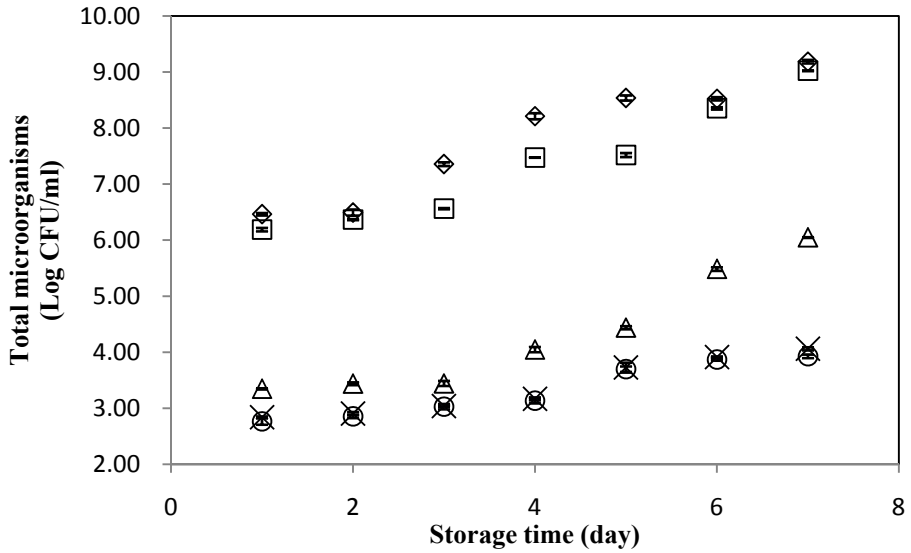

Figure 1. Microorganism concentration during storage of treated juice; control (◇); pasteurized (□); Filter A (△); Filter B (×); Filter C (○)

Microscopic evaluation showed the filamentous organisms which specifically indicates the presence of mold and fungi. Spores were also observed in the filaments. After 7 days of storage the total microorganisms of pasteurized juice showednon-significant difference to the control, which is the untreated sorghum juice. Table 1 shows the comparison of microbial content during storage.

Table 1: Microbial Content in Sorghum Juice After 7 Days Storage.

Treatment	Total mi**croorganisms** (x 10^6 CFU/ml)
Control	1540.0 ± 117.0^a
Pasteurized	1060.0 ± 17.7^a
Filter A	1.12 ± 0.01^b
Filter B	$11.60 \times 10^{-3} \pm 0.74 \times 10^{-3c}$
Filter C	$8.50 \times 10^{-3} \pm 0.64 \times 10^{-3c}$

Different superscripts a, b, c after the value show significant differences

Microfiltration process using pore size of 3.0 μm (Filter A) provided significant reduction of microorganism compared to pasteurization. After 7 days of storage the microbial load is 103 times

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lower than pasteurized juice. Microorganism size, especially of *Aspergillus* sp and *Rhizopus* sp is in the range of 2-5 μm (Gassem 1999; Reponen *et al*. 2001; Simões *et al*. 2013), therefore filtration using nominal pore size of 3.0 μm could be effective. However, smaller pores might be passed and growth was observed after 5 days of storage. Filtration effectiveness of Filter A was 99.93%.

In Filter B, filtration effectiveness was almost 100%. Sterility was not achieved because some microorganism such as coliform bacteria have width diameter of less than 0.2 μm and could passed through the filtration (Boone *et al*. 2013; Reshes *et al*. 2008). In Filter C, a serial of filter was installed to decrease the filtration load. Filter with pore size of 3.0 μm was expected to be effective for mold and fungi while filter with pore size of 1.0 μm served as bacterial removal. However, TPC analysis of sorghum juice after filtration using the series showednon-significant differences to Filter B. In laboratory scale, the prefiltration process showednon-significant difference in filter usage time. Pressure loss was also not significantly different. Blow melt polypropylene filters used in these experiments were designed to give low pressure loss due to lack of molecular interaction between solids and filter.

From the experiments it could be concluded that microfiltration with nominal pore size of 1.0 μm was sufficient for microbial removal in sorghum stalk juice.

3.2. Microbial Growth

The growth of *S. cerevisiae* was evaluated to indicate the suitability of treatment to the bioethanol production. Growth was monitored for 24 hours for all treatments and the results are shown in Fig 2 for aerobic growth and Fig 3 for anaerobic growth.

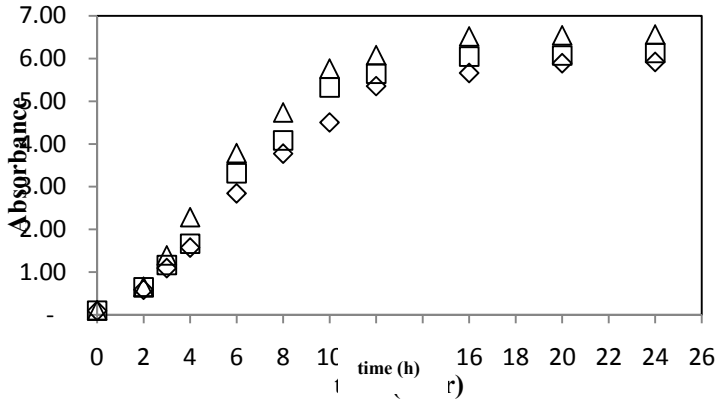

Figure 2. Microorganism growth in aerobic condition using sorghum juice as media; control (△); pasteurized (□); Filter B (◇)

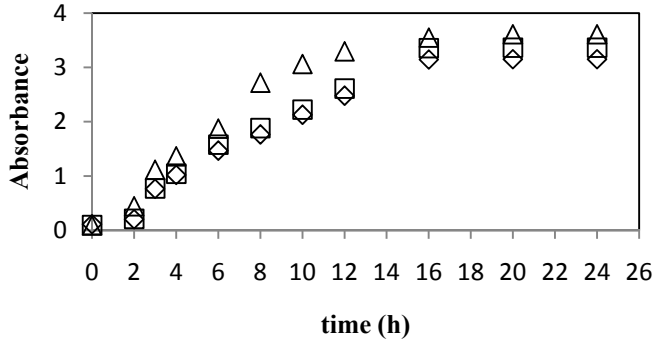

Figure 3. Microorganism growth in anaerobic condition using sorghum juice as media; control (△); pasteurized (□); Filter B (◇)

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Generally, there were no significant differences observed between control, pasteurized, and filtered juice. Method implied in this experiment gave total microbial load as turbidity, both of *S. cerevisiae* and the contaminant. Yeast *S. cerevisiae* inoculated is expected to be the dominant species, which later will suppress the contamination of other microorganism(Kundiyana *et al*. 2010). Doubling time of *Aspergillus* sp and *Rhizopus* sp is between 3.4-4.5 h while yeast doubling time recorded in the range of 1.5-1.7 h (Bitton 1998; Herskowitz 1988). Although the yeast dominates the growth, competition between *S. cerevisiae* with the contaminant was still observed due to limited nutrition and oxygen (Yuan *et al*. 2011).

In anaerobic condition the growth showed similar trend. Growth concluded at relatively similar cell concentration for all treatments. This might happen due to the death of growing spores after dissolve oxygen depleted. Several species of *Aspergillus* might grow at relatively low oxygen availability; however growth normally stops at oxygen level less than 0.1% (Hall & Denning 1994).

Table 2 shows the value of yield of cell to the substrate utilization ($Y_{X/S}$) of different treatments. This value indirectly shows the domination of microorganism in the reactor. Varied value of $Y_{X/S}$showed that consortiums of microorganism growing in the media were different.

Table 2: Yield of cell to substrate ($Y_{X/S}$) at treated sorghum juice

Treatment	$Y_{X/S}$Aerobic	$Y_{X/S}$Anaerobic
Control	0.44	0.17
Pasteurized	0.46	0.19
Filtration B	0.49	0.20

Filtration gave the value of $Y_{X/S}$ closest to the $Y_{X/S}$ of sterile sorghum juice, which are 0.67 (aerobic) and 0.26 (anaerobic). This indicated that after filtration, microbial domination established as expected and that the process was effective.

4. Conclusions

Microbial contamination removal is important to ensure fermentation in bioethanol production run with highest rate. From the study it could be concluded that filtration using micro filter with nominal pore size of 1.0 μm is effective for microbial contamination removal. Removal effectiveness was 99.9% and remaining microorganism did not grow to higher load after 7 days of storage. Growth and cell yield of *S. cerevisiae* cell in the treated juice was closer to the sterile condition. In comparison to pasteurization, microfiltration was proven to be more effective and requires lower energy. It is advisable to study the process using smaller pore size to ultrafiltration, however, the pressure loss and energy requirement for pumping might be increased.

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