Pol. J. Environ. Stud. Vol. 21, No. 4 (2012), 943-948

Original Research

Decolorization of Sugar Beet Molasses Vinasse, a High-Strength Distillery Wastewater, by Lactic Acid Bacteria

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Received: 28 August 2011
Accepted: 28 February 2012

Abstract

The color of sugar beet molasses vinasse is produced mainly by melanoidins (from Maillard reaction of sugars (carbohydrates) with proteins (amino groups)), caramels (from overheated sugars), and invert degradation products of alkaline hydrolysis. The aim of this study was to remove colorants from vinasse using lactic acid bacteria (*Lactobacillus coryniformis*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Weisella soli*, *Pediococcus parvulus*, and *Pediococcus pentosaceus*). The highest color removal was obtained with *L. plantarum* (44%). The highest reduction in melanoidins and invert degradation products was observed with *P. parvulus* (38% and 36%, respectively). The colorants underwent biotransformation. No correlation was found between color removal and COD reduction.

Keywords: colorant, melanoidins, distillery wastewater, decolorization, lactic acid bacteria

Introduction

The past decade has witnessed an increase in global bioethanol production, which in 2010 will total 85.9 billion liters compared to the 73.9 billion liters produced in 2009 [1, 2]. The production of one liter of ethanol is paralleled by the generation of 8 to 15 liters of high-strength wastewater [3] that is difficult to utilize.

Distillery wastewater is characterized by extremely high chemical oxygen demand (COD, 80-100 g/l), high biological oxygen demand (BOD, 40-50 g/l), low pH, an objectionable odor, and dark brown color [4, 5]. The color of the stillage is produced primarily by three groups of colorants: melanoidins (from Maillard reaction of sugars (carbohydrates) with proteins (amino groups)), caramels (from overheated sugars), and invert degradation products of alkaline hydrolysis [4]. Even though sugar beet molasses and sugar cane molasses stillage exhibit a similar dark brown color, the colorants have different origins. Those occurring in sugar cane molasses stillage are predominantly plant pigments associated with polysaccharides, whereas those found in sugar beet molasses stillage are alkaline degradation products of hexoses, melanoidins, and caramels [5, 6].

Wastewater has become a major concern, as it cannot be discharged into natural watercourses or a municipal sewage system. If such wastewater entered the environment, this would reduce photosynthetic activity and dissolved oxygen concentration, and thus severely affect aquatic plants and animals. Apart from the high COD, distillery wastewater also contains nitrogen, phosphorus, and potassium [7], which are to be blamed for the eutrophication of the aquatic environment. The disposal of distillery wastewater on land is equally harmful, as it reduces soil alkalinity, inhibits seed germination, and causes damage to vegetation [8].

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Because of its high colored matter content and high COD, distillery wastewater cannot be treated by conventional biological methods such as the activated-sludge process or anaerobic lagooning. Of the many physicochemical and biological processes that are in use, only a few have provided sufficient removal of colorants from cane molasses stillage [9-11]. So far, the ability of lactic acid bacteria to decolorize distillery wastewater has been studied only with *Lactobacillus hilgardii* [12], *Lactobacillus plantarum* [8], and *Lactobacillus casei* [13] in cane molasses wastewater. The available literature contains no references to the capacity of lactic acid bacteria for decolorizing sugar beet molasses vinasse. Decolorization of distillery wastewater generates value-added products (such as lactic acid biomass), and is an optional step in conventional ethanol production to move toward a biorefinery model production where all by- and waste products are utilized to increase the values produced and reduce waste production. This enables a cost-effective utilization of the problematic wastewater from ethanol [14].

The aim of this research was to remove colorants from a high-strength distillery wastewater, sugar beet molasses vinasse, using lactic acid bacteria.

Materials and Methods

Distillery Wastewater and Microorganisms

Sugar beet molasses vinasse samples were collected at the "CHEKO" Manufacturing Plant, Ltd, Włocławek, Poland, and were frozen at -20ºC before use. The pH and density of the wastewater was 5.02 and 22 °Blg, respectively. The liquid phase consisted of (g/l): chemical oxygen demand (COD), 94.1; total nitrogen (TN), 5.28; ammonia nitrogen (N-NH_4), 0.294; total phosphorus, 0.12; phosphate phosphorus, 0.069; reducing substances determined before hydrolysis, 7.58; reducing substances determined after hydrolysis, 12.09; glycerol, 9.71; glucose, 0.001; lactic acid, 18.14; propionic acid, 0.25; acetic acid, 2.07; pyroglutamic acid, 8.16; succinic acid, 1.46; valeric acid, 0.15; isobutyric acid, 5.95; tartaric acid, 0.8; products of alkaline degradation of invert sugars (PADIS), 20.07; caramels, 1.75, and melanoidins, 2.91.

Six bacterial strains (*Lactobacillus coryniformis*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Weisella soli*, *Pediococcus parvulus,* and *Pediococcus pentosaceus*) were obtained from the Department of Microbiology, Agricultural University of Uppsala, Sweden (a generous gift from Professor Johan Schnürer). The strains were stored in glycerol at -65ºC.

Inoculum

The volume for seeding was 0.1 ml of unfrozen suspension of each bacterial strain. The bacteria were inoculated under aseptic conditions into 100 ml MRS broth (De Man, Rogosa, Sharpe broth). The flask was incubated at 37ºC for 24 h before use in the experiments under static conditions. The suspension of 0.1 ml bacteria grown in the MRS broth was used as the inoculum.

Medium

MRS-agar medium (MRS-agar with 5% of sugar beet molasses vinasse) was used for the cultivation of lactic acid bacteria and for the screening of the strains.

Sugar beet molasses vinasse (25% v/v) with yeast extract (5.31 g/l), KH_2PO_4 (5 g/l), $MgSO_4 \cdot 7H_2O$ (0.75 g/l), and glucose (50 g/l)) was used for shake flasks experiments. Initial pH was adjusted to 6.25 with 30% NaOH. Glucose was added separately after sterilization of the stillage and supplements at 121ºC for 15 min.

Screening of Lactic Acid Bacteria

Strains with color removing ability were screened in a series of dilutions by the agar plate method. The diluted samples (0.1 ml) were spread onto the plates covered with the MRS-agar medium and incubated at 37ºC for one day. After that the colony-forming units (cfu/ml) were counted. The strains that had produced the largest quantity of colony-forming units were chosen for use in the next step. All experiments were conducted under aseptic conditions in triplicate. Average values were reported.

Process Conditions

Decolorization of the enriched sugar beet molasses vinasse by the strains chosen was carried out in 300 ml flasks (each containing 100 ml of the medium) at 37ºC and 120 rpm for 7 days. Samples were collected every 24 h. All experiments were conducted under aseptic conditions in triplicate. Average values were reported.

Analytical Methods

The samples were centrifuged at 8000g (Sigma® 4K15) for 15 min. The supernatant was stored for use in further analyses. Bacterial growth was monitored by measuring (at 620 nm) the absorbance of the cell suspension (diluted with 0.9% NaCl) obtained after centrifugation. Suspended solids (SS) were determined gravimetrically by drying the diluted cell suspension at 50ºC for 24 h and then at 105ºC until a constant weight was obtained.

Chemical oxygen demand (COD), total phosphorus (TP) and phosphate phosphorus (P-PO_4) were established spectrophotometrically using Dr. Lange cuvette tests [15]. Total nitrogen (TN) was determined by the Kjeldahl method. Ammonia nitrogen (N-NH_4) concentration was measured by distillation with water vapor in the Parnas apparatus. The concentrations of glucose, glycerol, and organic acids (lactic, acetic, propionic, pyroglutamic, succinic, malic, and isobutyric) were determined by HPLC (Knauer; UV-VIS and RI detectors; column type, Phenomenex ROA organic acids; column size, 7.8 mm i.d.×300 mm; effluent, 0.005 M H_2SO_4; flow rate, 0.5 ml/min; temperature, 40ºC).

Decolorization Yield

After centrifugation, the supernatant was diluted with distilled water and the diluted solution was analyzed for color intensity at 475 nm with a UV-VIS spectrophotometer. Decolorization activity was expressed as the difference between initial and final absorbance divided by initial absorbance [16]. The concentrations of melanoidins and caramels, as well as those of the products obtained from alkaline degradation of hexoses, were measured spectrophotometrically (at 250, 282, and 300 nm) and then made subject to calculations [17]. Colorants were also measured by HPLC (Knauer; detector UV-VIS; column type, Agela Unisol C18, 5 µm; column size, 4.6 mm i.d.×250 mm; effluent, 10% ACN/ 90% H_2O; flow rate, 0.5 ml/min; temperature, 27ºC). Detection wavelength was set to 290 nm [16].

Results and Discussion

Screening of Lactic Acid Bacteria for Decolorization

Four strains, *W. soli* ($1.2 \cdot 10^9$ cfu/ml), *P. pentosaceus* ($2.4 \cdot 10^9$ cfu/ml), *P. parvulus* ($1.4 \cdot 10^9$ cfu/ml), and *L. plantarum* (1.4·109 cfu/ml), were chosen for shake flask experiments as the microorganisms with a potential color removing ability. The strains *L. coryniformis* and *L. sakei* showed poorer growth ($2.5 \cdot 10^7$ cfu/ml and $1.9 \cdot 10^8$ cfu/ml, respectively).

Color and COD Removal

Every 24 h the extent of color removal (%) was determined by the difference in absorbance at 475 nm (Fig. 1).

While *L. plantarum* and *P. parvulus* decolorized the beet molasses vinasse from the first day of incubation, *P. pentosaceus* and *W. soli* caused the color of the effluent to increase (Fig. 1). From the second day on, all bacteria produced color removal, but the extent of removal was poor. The highest extent of decolorization was achieved on day 4 with *L. plantarum* (44%), followed by *P. parvulus* (41%), and the lowest extent with *W. soli* (10%). Similar results were reported by Tondee and Sirianuntapiboon [8] who

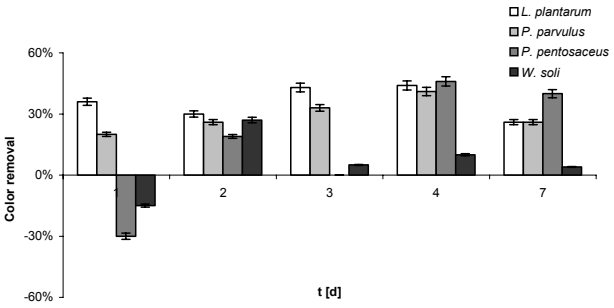

Fig. 1. Decolorization of sugar beet molasses vinasse by the bacterial strains screened on days 1, 2, 3, 4, and 7 of the process [%]. Error bars represent the standard deviation of replicate (n=3).

decolorized cane molasses with *L. plantarum* No. PV71-1861. In their studies the highest extent of color removal amounted to 55.3% and was achieved within 7 days at a concentration of colorants that was only one-fifth the concentration used in our present studies. Similar color removal was obtained by Shibu et al. [13] within 5 days of decolorization of anaerobically digested cane molasses stillage by *L. casei*. In their studies, which involved three different concentrations of stillage in the medium (10, 20, and 30%, v/v)), the highest extent of color removal totaled 52%, which was more than we obtained in the present study. However, in our study, stillage concentration in the medium was 2.5 times the lowest concentration used by Shibu et al. [13]. With the other two concentrations, 20% and 30%, they reached an extent of color removal amounting to 35% and 27%, respectively. And this indicates that with almost the same concentration of stillage in the medium (25%) the *L. plantarum* strain used in our study produced a higher extent of decolorization, which totaled 44%. Our study also produced higher decolorization values compared to those reported by Ohmomo et al. [12], who achieved color removal of 28% with *Lactobacillus hilgardii* W-NS.

In the present study the maximal removal of chemical oxygen demand (COD) was low for each of the strains tested. With *P. parvulus*, *L. plantarum*, and *P. pentosaceus*, the reduction in COD did not exceed 3%. What is more, *W. soli* caused the COD to increase by approx. 5%. As can be noticed, the removal of colorants is not correlated with the removal of organic matter. In contrast to the efficiency of COD reduction achieved in our study, Shibu et al. [13] attained COD removal of 57% with *L. casei* within 5 days of batch decolorization of 10% v/v digested molasses spentwash. When the concentration of stillage in the medium was higher (20% and 30% v/v), COD reduction was lower: 46% and 38%, respectively [13]. The increase in COD content and the low COD removal might be associated with enhanced synthesis of organic acids (Tables 1). Ohmomo et al. [12] suggested that degradation of melanoidins was concomitant with the synthesis of lactic acid. This implies that decolorization could be attributed to a reaction induced by a secondary metabolite. Color degradation coincides with changes in molecular structure, but total mineralization of organic matter does not seem to occur [18]. The increase in COD might well be associated with the removal of betaine (one of the sugar beet molasses vinasse components). Although dichromatic analysis of COD fails to detect betaine [19], it detects the metabolites of betaine biodegradation, and this may be the reason why COD increased with the progress of the process.

Removal of Products of Alkaline Degradation of Invert Sugar (PADIS), Caramels, and Melanoidins

The removal of colorants (PADIS, caramels, and melanoidins) with time is depicted in Fig. 2.

As can be seen, all of them have been removed only by *L. plantarum* and *P. parvulus*. With *W. soli*, the content of PADIS and melanoidins increased, while that of caramels

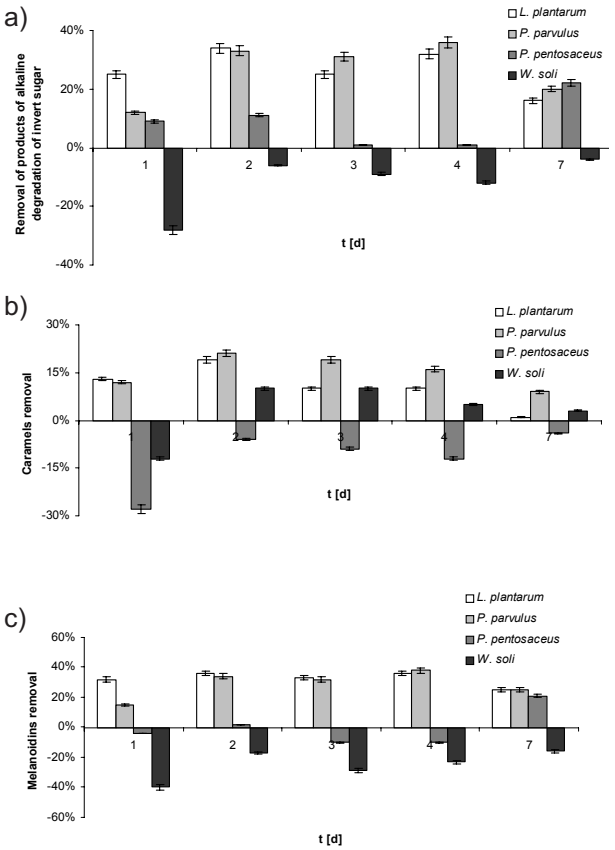

Fig. 2. Removal of alkaline products of invert degradation (a), caramels (b), and melanoidins (c) on days 1, 2, 3, 4, and 7 of the process [%].
Error bars represent the standard deviation of replicate (n=3).

did not. Maximum reduction in PADIS and melanoidins was observed on day 4 of the experiment with *P. parvulus* (36% and 38%, respectively); similar reduction efficiencies were achieved with *L. plantarum* (34% and 36%). Maximum removal of caramels was attained on day 2 with *P. parvulus* and *L. plantarum* (21% and 19%, respectively).

HPLC Analysis

HPLC analysis of degraded samples after 4 days of the process has revealed a reduction in peak height (retention time = 4.9 min) as compared with the control sample (Fig. 3).

The highest reduction in the main peak was observed with *L. plantarum* (57.9 %) and *P. pentosaceus* (45.5 %), which are the strains that also showed the greatest color removing ability (Fig. 1). However, the highest reduction in the peak areas occurred with *P. parvulus* (42.0 %) and *P. pentosaceus* (40.0 %), as well as with *L. plantarum* and *W. soli* (29.3% and 7.4%, respectively). The results achieved in this study corroborated those obtained by Bharagava and Chandra [20] and Bharagava et al. [16]. Their HPLC analysis of degraded melanoidins samples has disclosed smaller peaks compared to the control sample. Bharagava et al. [15] also suggested that the decrease in color intensity might be largely attributed to the bacterial degradation of melanoidins by the bacterial peroxidase enzymatic reaction.

The bacterial strains examined in our present study assimilated glucose, as well as tartaric, acetic, and pyroglutamic acids, to produce isobutyric and lactic acids (Table 1).

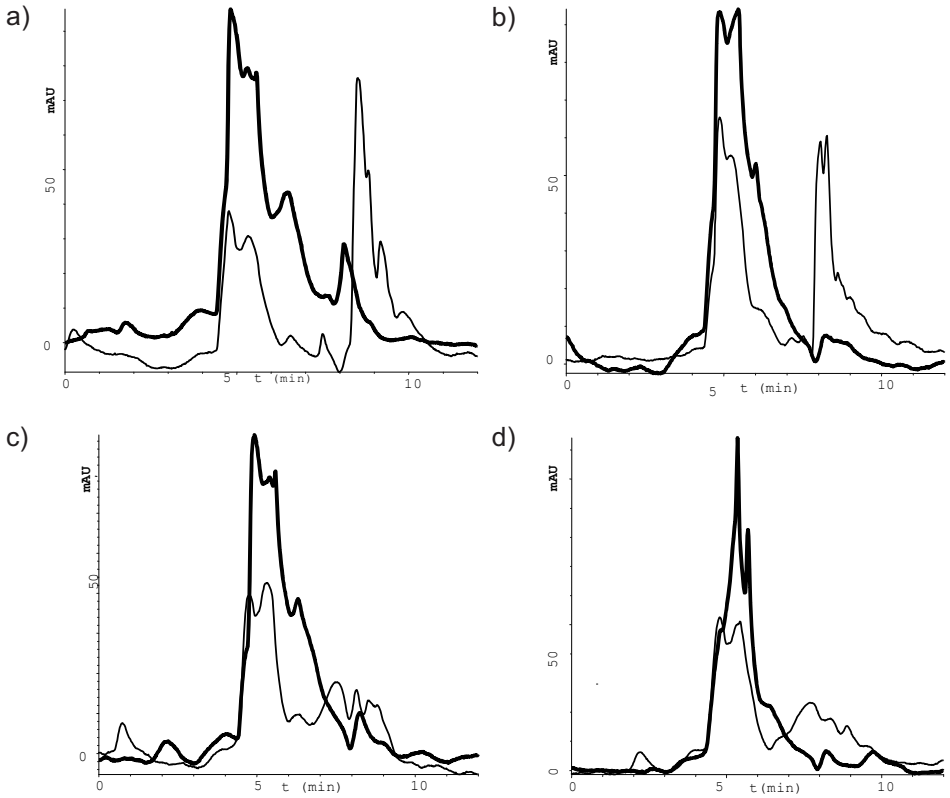

Fig. 3. HPLC chromatogram of control (thick line) and degraded samples (thin line) after 4 days of incubation: *L. plantarum* (a), *P. parvulus* (b), *P. pentosaceus* (c), and *W. soli* (d).

Table 1. Removal of organic acids and glucose on day 4 of the process [%].

Substance / Strain	*L. plantarum*	*P. parvulus*	*P. pentosaceus*	*W. soli*
Tartaric acid	9.68 (±0.02)	17.8 (±0.06)	20.41 (±0.08)	68.42 (±0.04)
Acetic acid	100 (±0)	100 (±0)	100 (±0)	100 (±0)
Pyroglutamic acid	9.60 (±0.53)	17.55 (±0.27)	1.94 (±0.11)	1.71 (±0.05)
Isobutyric acid	-8.37 (±0.16)	-6.91 (±0.34)	-102.40 (±0.15)	-18.40 (±0.06)
Lactic acid	-106.02 (±0.36)	-78.85 (±0.76)	-94.97 (±0.33)	-48.42 (±0.16)
Glucose	48.86 (±0.47)	38.80 (±0.34)	36.64 (±0.57)	46.13 (±0.59)

Negative values indicate increase in content.

The highest increase in the content of lactic acid (mg/ml) was observed with *L. plantarum* (5.56 g/l), and the lowest with *W. soli* (2.88 g/l). The quantity of lactic acid produced was lower than that obtained by Shibu et al. [13] on day 5 of batch fermentation of digested molasses spentwash (30% v/v) by *L. casei* (8.3 g/l). The assimilation of acetic acid was complete in all of the experiments. The utilization of glucose was incomplete, which accounts for the poor lactic acid synthesis. The low yield of lactic acid may also be attributed to the uncontrolled pH.

Biomass Growth

The highest biomass growth was obtained on day 7 of the process for all of the strains examined. The increment in biomass was the highest with *P. parvulus* (88%), followed by *P. pentosaceus* (86%) and *W. soli* (75%) (Fig. 4).

The highest increase in suspended solids content was observed with *L. plantarum* (1.5 g/l), followed by *P. pentosaceus* (1.3 g/l).

Conclusions

Among the six strains of lactic acid bacteria examined for potential color removing ability, *Lactobacillus plantarum* produced the highest color removal (44%) after 4 days of cultivation. The highest reduction in melanoidins, as well as in the products of alkaline degradation of invert sugar, was observed on day 4 with *Pediococcus parvulus* (38% and 36%, respectively). Similar color reduction values for melanoidins and invert degradation products were achieved with *L. plantarum* (36% and 34%, respectively). Spectrophotometric and HPLC analyses of treated wastewater have demonstrated that the colorants underwent biotransformation. No correlation was found to occur between color removal and COD reduction.

All of the findings substantiate the choice of the strain *L. plantarum* for further studies on the environment-friendly removal of colorants from distillery wastewater. Microbial decolorization of distillery effluents shows great promise as a cost-effective, environmentally-safe biotechnology for the treatment of high-strength industrial wastewater. Future research should address the following major issues: supplementation of different carbon sources, optimization of the amounts added, and the need to dilute the stillage being decolorized.

Acknowledgements

Professor Johan Schnürer (Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden) is acknowledged for his generous gift of the LAB samples.

Ryszard Chojnacki (CHEKO Manufacturing Plant, Ltd, Włocławek, Poland) is acknowledged for the distillery wastewater samples.

This study was financed by the National Science Centre (Poland) under Project No. N N312 421940.

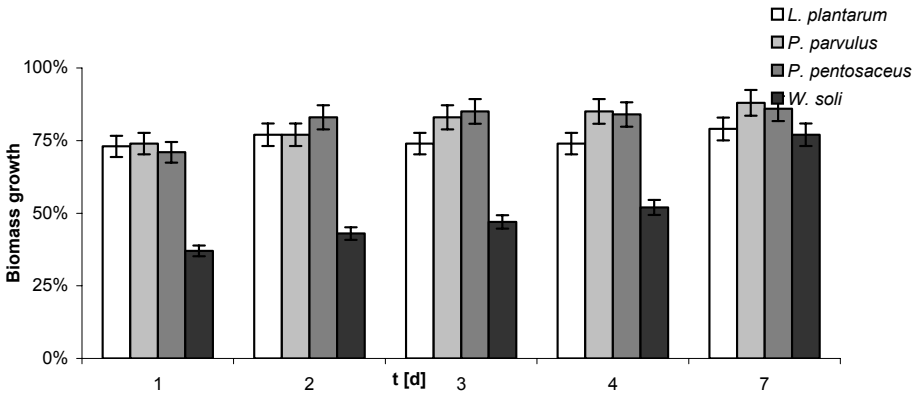

Fig. 4. Biomass growth after days 1, 2, 3, 4, and 7 of the process [%] (absorbance measured at 620 nm).

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