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Original Research

Screening of Medium Components and Process Parameters for Sugar Beet Molasses Vinasse Decolorization by *Lactobacillus plantarum* Using Plackett-Burman Experimental Design

Przemysław Seruga, Małgorzata Krzywonos*

Department of Bioprocess Engineering, Wrocław University of Economics,
Komandorska 118/120, 53-345 Wrocław, Poland

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Abstract

Sugar beet molasses vinasse is characterized by a high COD and dark brown color produced by three groups of colorants: melanoidins, caramels, and invert degradation products of alkaline hydrolysis. These environmental pollutants have to be effectively removed *via* biodegradation processes before discharge into a watercourse. The aim of this study was to assess how the type of the bacterial culture used (static or agitated), and the addition of nutrients (wheat stillage, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$, KH_2PO_4, yeast extract, glucose, fructose, sucrose, and peptone) contribute to the effectiveness of sugar beet molasses vinasse decolorization with *Lactobacillus plantarum*. The Plackett-Burman experimental design was used to reduce the number of experiments. Decolorization of nutrient-enriched vinasse (25% v/v) was performed at an initial pH of 6.25 and 37ºC for 7 days. Color was measured spectrophotometrically at 475 nm. Concentrations of sugars, organic acids, and colorants were determined by HPLC. The nutrient was screened when the probability value exceeded 95% of the confidence level. 67% efficiency of decolorization of sugar beet molasses vinasse by *L. plantarum* was obtained. The type of bacterial culture and the addition of wheat stillage, yeast extract, glucose, peptone, and $(NH_4)_2SO_4$ were significant factors for the decolorization process.

Keywords: decolorization, vinasse, melanoidins, *Lactobacillus plantarum*, Plackett-Burman experimental design

Introduction

Sugar beet molasses vinasse, a by-product of ethanol production, has dark brown color, which is due to the presence of colored compounds formed in sugar beet processing as a result of sugar degradation, pH changes, thermal effects, and chemical reactions [1]. The colorants present in the vinasse can be classified into three groups: melanoidins (formed in Maillard reaction), caramels (products of sugar degradation at high temperature), and invert degradation products of alkaline hydrolysis [1, 2]. The presence of melanoidins is the main contributing factor in the dark brown color, which persists in the vinasse even after anaerobic treatment and is difficult to remove in extended aeration tanks [3].

Vinasse cannot be discharged directly into natural water systems [4]. Various physico-chemical and biological processes have been applied to the treatment of distillery effluents, but only a few provided ample color reduction from cane molasses stillage [5-7]. Color removal from cane

*e-mail: malgorzata.krzywonos@ue.wroc.pl

molasses wastewater by lactic acid bacteria has been studied with *Lactobacillus hilgardii* [8], *Lactobacillus plantarum* [9, 10], and *Lactobacillus casei* [11]. Sugar beet vinasse was used for color removal in only one study, when bacteria *Lactobacillus coryniformis, L. sakei, L. plantarum, Weisella soli, Pediococcus parvulus,* and *P. penthosaceus* were tested [12]. Ohmomo et al. [8] achieved the decolorization efficiency of 12.3% after the 1st day of cultivation and 19.2% after the 3rd day. Higher color removal of 49%, 35%, and 27% was reported by Shibu et al. [11] within 5 days of fermentation for 10, 20, and 30% vinasse (v/v), respectively. *L. plantarum* showed melanoidin pigment removal yield of 68.12% in 7 days experimental time for anaerobic-treated molasses wastewater [9]. Decolorization of sugar beet molasses vinasse by lactic acid bacteria was studied in our previous study, when among six examined strains, for *L. plantarum* we achieved highest color removal (44%) [12].

The aim of this study was to assess how the type of bacterial culture used (static or agitated) and the addition of nutrients (wheat stillage, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$, KH_2PO_4, yeast extract, glucose, fructose, sucrose, and peptone) contribute to the effectiveness of sugar beet molasses vinasse decolorization with *Lactobacillus plantarum*. The Plackett-Burman experimental design was used to reduce the number of experiments [13].

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 22ºBlg, respectively. The liquid phase is characterized in Table 1.

Lactobacillus plantarum MiLab93 was obtained from the Department of Microbiology, Agricultural University of Uppsala, Sweden. The strain was stored in glycerol at -65ºC.

Table 1. Characterization of the liquid phase of sugar beet vinasse.

Parameter	Value [g/l]
Chemical oxygen demand (COD)	94.1
Total nitrogen (TN)	5.28
Ammonia nitrogen ($N\text{-}NH_4$)	0.294
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	20.07
Caramels	1.75
Melanoidins	2.91

Inoculum

The volume for seeding was 0.1 ml of unfrozen suspension of 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.

Plackett-Burman Statistical Design

The Plackett-Burman experimental design was used to assess how the type of the bacterial culture used (static or agitated) and the addition of nutrients (wheat stillage, $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$, KH_2PO_4, yeast extract, glucose, fructose, sucrose, and peptone) contribute to the effectiveness of sugar beet molasses vinasse decolorization with *Lactobacillus plantarum* (Table 2).

The minimal and maximal levels (denoted by (-1) and (1) respectively) selected for the 10 factors are presented in Table 3.

The results of the experimental design were analyzed and interpreted using STATISTICA version 10 (StatSoft, Inc., 2011, USA) statistical software.

Process Conditions

Decolorization of the enriched sugar beet molasses vinasse (Table 2) was carried out in 300 ml flasks (each containing 100 ml of the medium) at 37ºC for 7 days. Samples were collected every 24 h. All experiments were conducted under aseptic conditions in duplicate. Average values are reported.

Analytical Methods

The samples were centrifuged at 8,000 g (Sigma® 4K15) for 15 min. The supernatant was stored for use in further analyses. Chemical oxygen demand (COD), total organic carbon (TOC), total phosphorus (TP), and phosphate phosphorus ($P\text{-}PO_4$) were established spectrophoto-

Table 2. Plackett-Burman experimental design matrix for 10 factors.

Run	Factor									
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}
1	1	-1	1	-1	-1	-1	1	1	1	-1
2	1	1	-1	1	-1	-1	-1	1	1	1
3	-1	1	1	-1	1	-1	-1	-1	1	1
4	1	-1	1	1	-1	1	-1	-1	-1	1
5	1	1	-1	1	1	-1	1	-1	-1	-1
6	1	1	1	-1	1	1	-1	1	-1	-1
7	-1	1	1	1	-1	1	1	-1	1	-1
8	-1	-1	1	1	1	-1	1	1	-1	1
9	-1	-1	-1	1	1	1	-1	1	1	-1
10	1	-1	-1	-1	1	1	1	-1	1	1
11	-1	1	-1	-1	1	1	1	1	-1	1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

metrically using Dr. Lange cuvette tests [14]. Total nitrogen (TN) was determined by the Kjeldahl method [15]. Ammonia nitrogen ($N\text{-}NH_4$) concentration was measured by distillation with water vapour in the Parnas apparatus [15]. 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 sample centrifugation, the supernatant was diluted with distilled water. The diluted solution was analyzed for color intensity at 475 nm with a UV-VIS spectrophotometer.

Decolorization efficiency 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]. Melanoidin standards have been prepared by diluting glucose (4.5 g), glycine (1.88 g), and $NaHCO_3$ (0.42 g) in 100 ml distilled water and heating for 7 hours at 95ºC. During the process water was added [18].

Results and Discussion

Plackett-Burman Statistical Design

The Plackett-Burman model was used to evaluate the influence of 10 factors on sugar beet vinasse decolorization. The design for 12 runs with two levels for each factor is shown in Table 2. Maximal color removal (67%) was obtained during run 8 with maximal levels of glucose, fructose, sucrose, KH_2PO_4, and peptone concentrations, and maximal level of rpm range (Table 2 and Fig. 1) followed by decolorization yield of 36% observed in run 12 with minimal levels for factors. Chemical oxygen (COD) removal was very low, what is more, in run 7 and 10 it was increased in COD. Maximum extent in COD was observed in run 8, the same run when maximum color removal was achieved (Fig. 1). Color removal was not correlated with COD reduction. Pearson correlation coefficient for these two variables amounted to 0.0096. Obtained results corroborated with our previous study [12], when COD removal was not correlated with the removal of colorants.

Similar color removal efficiency (55.3%) was reported by Tondee and Sirianuntapiboon [9], who decolorized anaerobic-treated cane molasses with *L. plantarum* No. PV71-1861. These values were achieved within 7 days at a concentration of colorants that were only one-fifth the concentration used in our present studies. Shibu et al. [11] decolorized anaerobically digested cane molasses stillage with *L. casei* and three concentrations of stillage in the medium: 10, 20, and 30% (v/v) to obtain the highest extent of color removal (52%) at the lowest concentration (10%) within 5 days of the process. With the other two concentrations, 20% and 30%, they reached an extent of color

Table 3. Factor levels and assigned parameters in Plackett-Burman design.

Factor	Parameter	Minimal level	Maximal level
X_1	Wheat stillage (%v/v)	0	75
X_2	Yeast extract (g/l)	5	50
X_3	Glucose (g/l)	5	50
X_4	Fructose (g/l)	5	50
X_5	Sucrose (g/l)	5	50
X_6	$MgSO_4 \cdot 7H_2O$ (g/l)	0	7.5
X_7	KH_2PO_4 (g/l)	5	50
X_8	Peptone (g/l)	5	50
X_9	$(NH_4)_2SO_4$ (g/l)	0	4.5
X_{10}	Agitation (rpm)	0	120

removal amounting to 35% and 27%, respectively. Ohmomo et al. [8] reported an extent of color removal totaling 28%, which was attained with *Lactobacillus hilgardii* W-NS. Achieved color removal efficiency of 67% is also higher than that reported in our previous study [12]. The extent of decolorization was achieved on day 4 with *L. plantarum* (44%) followed by *P. parvulus* (41%), and it was similar to that obtained during run 12 of the current study. Based on that it can be assumed that some investigated factors have positive impact on the effectiveness of sugar beet molasses vinasse decolorization.

Table 4 compiles the effect, t ratio, and P value of the Plackett-Burman design. Wheat stillage (X_1), yeast extract (X_2), glucose (X_3), peptone (X_8), $(NH_4)_2SO_4$ (X_9), and agitation (X_{10}) were found to be significant for the decolorization process. Impacts of these factors on LAB cell growth and lactic acid production were reported in studies on selection of medium components for LAB [19-21].

Other factors, e.g. sucrose, $MgSO_4 \cdot 7H_2O$, and KH_2PO_4, were found to be insignificant (Table 4). Based on this it can be stated that vinasse contains sufficient concentrations of $MgSO_4 \cdot 7H_2O$ and KH_2PO_4, and there was no need for the addition of those nutrients. Medium enrichment with fructose and sucrose also was not necessary. Carbon source as added glucose was enough for cultivation.

The determination coefficient (R^2) of 0.91 (which means that 91% of the total variation is explained by the model) suggests a satisfactory representation of the process model and a good correlation between the experimental and predicted values [22, 23]. We therefore propose a linear equation describing the response of the decolorization yield:

$$Y = 0.428 - 0.0072X_1 - 0.0978X_2 + 0.063X_3 + 0.0419X_8 - 0.0967X_9 - 0.0029X_{10}$$

...where Y is predicted response and X_1, X_2, X_3, X_8, X_9, and X_{10} are coded values of the factors in Table 3.

The positive coefficients for glucose and peptone suggest a linear effect on the increment in decolorization, while the negative coefficients for other factors imply a linear effect on the decrement in color removal. This statement can be confirmed by results of run 12 (with all factors in minimum level) and low decolorization yields when the medium contains wheat stillage and yeast extract.

HPLC Analysis

HPLC analysis revealed a reduction in the peak areas of the samples decolorized after four days of cultivation, in comparison with the control (Table 5).

The highest reduction in peak area was observed in run 12 (67.3%) and run 8 (63.5%). These are also the runs for which the highest extent of decolorization was determined (Fig. 1). The highest reduction in peak height (retention time RT = 4.9 min, which was characteristic for prepared melanoidins standard: glucose-glycine) was noticed in run 12 (69.9%) and run 3 (50.1%). In run 8, the extent of reduction amounted to 40.3% (Table 5). Bharagava et al. [16] and Krzywonos and Seruga [12] used HPLC analysis to determine the removal of melanoidins and found that the degraded samples of melanoidins showed lower peak heights compared to the control sample. Bharagava et al. [16] also suggested that the decrease in color intensity might be largely attributed to the bacterial degradation of melanoidins by the bacterial enzymatic reaction.

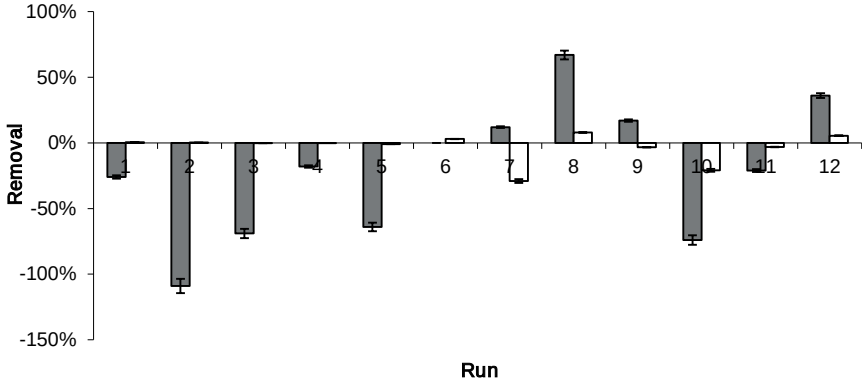

Fig. 1. Color (grey) and COD (white) removal.

Table 4. Effect estimates for color reduction of Plackett-Burman design.

Factor	Parameter	Effect	t-ratio	P value	Significance*
X_1	Wheat stillage	-0.0072	-6.2380	0.00003	+
X_2	Yeast extract	-0.0978	-5.0516	0.00022	+
X_3	Glucose	0.0630	3.2529	0.00629	+
X_4	Fructose	0.0244	1.2629	0.22881	-
X_5	Sucrose	-0.0030	-0.1531	0.88069	-
X_6	$MgSO_4 \cdot 7H_2O$	0.1867	1.6073	0.13199	-
X_7	KH_2PO_4	0.0159	0.8228	0.42546	-
X_8	Peptone	0.0419	2.1622	0.04983	+
X_9	$(NH_4)_2SO_4$	-0.9667	-4.9942	0.00025	+
X_{10}	Agitation	-0.0029	-4.0183	0,00146	+

*Significant at 5% level ($P \leq 0.05$), standard error = 0.213, $R^2 = 0.91$

Table 5. Reduction in peak area, peak height, and glucose content after four days of process (a minus sign in front of the number denotes an increase).

Run	Peak height reduction [%]	Peak (RT=4.9 min) area reduction [%]	Glucose removal [%]
1	45.8	36.3	37.0
2	33.1	46.7	66.5
3	50.1	30.6	74.5
4	20.4	31.2	67.5
5	42.6	47.8	35.0
6	-1.5	6.8	56.0
7	-44.9	-55.4	70.7
8	40.3	63.5	96.1
9	27.3	1.2	30.0
10	-2.3	-3.7	100
11	-38.9	-25.4	100
12	69.9	67.3	100

Glucose was main sugar used for fermentation. Reduction in glucose concentration varied markedly, ranging between 30% and 100% in all of the experiments (Table 5). Runs 8, 10, 11, and 12 provided efficiencies of glucose assimilation higher than 96%.

Conclusion

The highest color removal (67%) was obtained in run 8 with the maximum levels of glucose, fructose, sucrose, KH_2PO_4, and peptone concentrations, and with the maximum level of the rpm range. However, results of run 12 with minimum levels of factors and reduction in glucose concentration can prove that medium enrichment with fructose and sucrose was not necessary. Color removal was not correlated with COD reduction. The type of bacterial culture and the addition of wheat stillage, yeast extract, glucose, peptone, and $(NH_4)_2SO_4$ were significant for the decolorization process. Optimization of values for a significant factor should be investigated in future research.

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