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Microbiological Colourants Removal from Sugar Beet Molasses Vinasse – The Effects of Process Parameters and Vinasse Dilution

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Abstract: Distilleries, in addition to ethanol, produced vinasse which is hazardous for the environment. Sugar beet molasses vinasse (BMV) is the most problematic waste from distilleries because of the coloured compounds contained therein. Traditional methods of the removal of the pollutant load from the waste do not allow simultaneous decolourization. The paper presents a microbiological method of coloured compounds removal from BMV. The conditions of the process (pH and temperature) and vinasse concentration were optimized. The bacteria *Lactobacillus plantarum* MiLAB393 applied showed the decolourization activity of 26% in medium consisted of 30% v/v of BMV at pH0=6.5 and 35.8°C.

Keywords: decolourization, sugar beet molasses vinasse, *Lactobacillus plantarum*, lactic acid bacteria

JEL codes: Q16, Q53, Q57

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1. Introduction

In the agricultural distilleries where the raw material used to produce ethanol is sugar beet molasses, in addition to the main product, sugar beet molasses vinasse is also formed. It is a by-product with a high load of pollutants (COD=55.5-147 g $O_2 \cdot dm^{-3}$, BOD_5=27.5-69.3 g $O_2 \cdot dm^{-3}$), acidity, pH=5.0 and dark brown color (Ryznar-Luty et al., 2009; Vlissidis, Zouboulis, 1993;

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Vlyssides, 1997; Wilkie et al., 2000). It is believed that pollution from distilleries is among the most disturbing threats that humanity faces to date (Santal et al., 2016).

Under the influence of the conditions in the treatment processes of sugar beet juice and later of molasses in ethanol production, coloured compounds such as melanoidins, caramel and invert degradation products of alkaline hydrolysis (IDPAH) are formed. Dark brown vinasse can not be discharged into the surface water because this would distort the photosynthesis processes thereby affecting the functioning of the aquatic ecosystems. BMV disposal on the fields also has its limitations because of the composition of vinasse which results in it only being a possibility of growing exclusively non-food crops. BMV, in contrast to other vinasse, can not be used as an additive to animal feed in an amount greater than 2%. Besides, the cattle will not eat the vinasse in the spring and summer. The condition of this how to dispose of BWM is also the location of farms, since the transport of waste over a distance of more than 10 km is economically unprofitable. To use vinasse to produce biogas and then as fertilizer seems also interesting. A disadvantage is the need to invest in the construction of tanks for storing the effluent from biogas. This investment is necessary due to the seasonality of fertilizing the fields. In addition, the tanks occupy an area that could be used for agricultural production, which creates an opportunity cost in the form of lost profits from this production. The scale of the problem of managing the distillation waste is enormous, because for every one dm^3 of ethanol produced, 10 to 15 dm^3 of vinasse is generated (Szoege and Wiśniewski, 2013; Kumar et al., 1997).

Currently, there are attempts to decolourize vinasse by both physicochemical (Arimi et al., 2015; Bernal et al., 2016) and microbiological (Agnihotri, 2015; David et al., 2015; Georgiou et al., 2016; Santal et al., 2016) methods. The present study proposes the use of lactic acid bacteria, which occur naturally in the sugar beet molasses vinasse, and after decolourization, they could assist the preparation process of silage feed with the addition of purified vinasse.

2. Materials and methods

Microorganism. *Lactobacillus plantarum* MiLAB393 was obtained from the Department of Microbiology, the Swedish Agricultural University of Uppsala. The strain was stored in the MRS medium (de Man, Rogosa and Sharpe; Biocorp) with 10% v/v glycerol, at -65°C.

Inoculum. The unfrozen bacterial cell suspension, the volume of 0.1 cm^3, was activated in 100 cm^3 of sterile MRS medium. The culture was incubated at 37°C for 72 h under static conditions. After this time, the culture medium was inoculated with 1 cm^3 of a bacteria suspension in MRS medium, which corresponded to 1.5 $g \cdot dm^{-3}$ of bacteria dry weight.

Vinasse. The sugar beet molasses vinasse was collected from the CHECO Manufacturing Plant, Ltd., Włocławek, Poland, whose characterisation is shown in table 1.

Table 1. Vinasse characterization

Parameter	Value*
pH	5.0
COD	89.3 ± 4.8
BOD_5	256 ± 10.7
glycerol	3.9 ± 0.19
glucose	1.42 ± 0.07
total nitrogen	5.075 ± 0.205
total phosphorus	0.1 ± 0.02
lactic acid	20.4 ± 1.02
acetic acid	2.22 ± 0.11
pyroglutamic acid	8.51 ± 0.43
succinic acid	11.65 ± 0.58
isobutyric acid	21.07 ±1.05
tartaric acid	1.04 ± 0.05
gluconic acid	2.17 ± 0.11
invert degradation products of alkaline hydrolysis	20.07 ± 1
caramels	1.75 ± 0.09
melanoidins	2.91 ± 0.15

* all values except pH are expressed in $g \cdot dm^{-3}$

Source: own elaboration

The absorbance of BMV measured at a wavelength of 475 nm was 10.48. Measurements were made for dilution while maintaining a linear relationship function A=f (c).

Process condition. The process was optimized using the method of experiment planning and analysis of variance (ANOVA), the module of Industrial Statistics, Design of Experiments (DOE) (StatSoft, Inc., 2011). The Box-Behnken plan with three input quantities was applied: pH (X_1), the temperature (X_2) and the vinasse concentration (X_3). The plan called for the execution of the 15 variants of the experiments in duplicate, with variance encoded on three-levels (table 2). Encoded input variables assigned to the applied experimental plan as given in table 3. The optimization criterion (response function) was the degree of vinasse decolourization (Y%).

Table 2. Coded variables in the matrix of the experiment

Variant	(X_1)	(X_2)	(X_3)
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0

Source: own elaboration.

Table 3. Values assigned to the plan

	X_1	X_2	X_3
-1	4,0	23	5
0	5,5	30	25
1	7,0	37	45

Source: own elaboration.

The mathematical model of experimental plan with 3 variables is expressed by the following equation:

$$Y = \beta_0 + \sum\beta_1 X_1 + \sum\beta_2 X_2 + \sum\beta_3 X_3 + \sum\beta_{1i} X_1^2 + \sum\beta_{2i} X_2^2 + \sum\beta_{3i} X_3^2$$

where:

Y – optimisation measure (response function, the degree of decolourization [%]);

X_1, X_2, X_3 – encoded values;

β_0, β_1, β_2, β_3, β_{1i}, $\beta_{2i,}$ β_{3i}, – model coefficients.

Fit of the model is expressed by a coefficient of determination R^2, and the statistical significance was tested using the Fisher test (F). The statistical significance of the regression coefficients was evaluated by Student test (t). The processes of decolourization were carried out by shake-flasks culture (120 rpm) for four days. Erlenmeyer flasks (250 cm^3) were filled up with 100 cm^3 of culture

medium supplemented with glucose and yeast extract at a dose rate [g dm^{-3}]: 18.09 and 3.37, respectively (Wilk et al., 2015). The carbon source was added to the medium after sterilization (121°C for 15 minutes). Temperature, pH and BMV concentration were set according to the plan of the experiment. The pH of the medium was determined using 33% NaOH. Samples were collected every 24 h.

Analytical methods. Samples of BMV before analysis were centrifuged using a laboratory Sigma 4K15 centrifuge at a speed of 8000 *g* for 15 min.

Bacterial growth was determined spectrophotometrically (DR 5000, Hach) at 620 nm. The degree of decolourization was determined by measuring the absorbance of the supernatant at 475 nm (Bharagava et al., 2009). The degrees of reduction in the concentrations of melanoidins, caramel and IDPAH were determined by Ivanov-Sapronov method (Sapronov, 1963; Krzywonos et al., 2016). Chemical oxygen demand (COD) was established spectrophotometrically using Hach Lange cuvette tests (Anonymous, 2000). Organic acids and glucose 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; eluent, 0.2 mM H_2SO_4; effluent, 0.5 cm·min^{-1}; temperature, 65°C, λ=210 nm).

The effectiveness of decolourization, the degree of COD and other components and indicators of vinasse reduction (% A) were calculated from the formula:

$$\%A = \frac{(A_0 - A_t)}{A_0} \cdot 100\%$$

where:

A_0 – the initial value of the absorbance/concentration of the compound or indicator,

A_t – value of the absorbance/concentration of the compound or indicator in the time *t*.

3. Results and Discussion

Statistically, the most significant effect ($p \leq 0.05$) for the decolourization process using a strain of *L. plantarum* MiLAB393 was vinasse concentration and the process temperature. The pH was not statistically significant (table 4 and 5).

Table 4. Regression model coefficient data

Factor	Effect	Standard error	t	p	-95% Confidence level	+95% Confidence level	Regression coefficient
Constants	**-22.9255**	**5.39076**	**-4.25273**	**0.000300**	**-34.0771**	**-11.7738**	**-22.9255**
X_1	12.0352	13.20462	0.91144	0.371517	-15.2806	39.3511	6.0176
X_1^2	0.5586	9.71834	0.05748	0.954663	-19.5453	20.6625	0.2793
X_2	**106.7865**	**13.20462**	**8.08706**	**0.000000**	**79.4707**	**134.1023**	**53.3933**
X_2^2	**32.3291**	**9.71834**	**3.32660**	**0.002936**	**12.2252**	**52.4330**	**16.1645**
X_3	22.7433	13.20462	1.72237	0.098425	-4.5726	50.0591	11.3716
X_3^2	**22.6483**	**9.71834**	**2.33047**	**0.028913**	**2.5444**	**42.7522**	**11.3242**

Bolded values $p \leq 0.05$
Source: own elaboration.

Table 5. Analysis of variance for the fitted model

Factor	Sum of squares	df	Mean sum of squares	F - value	p
X_1	579.39	1	579.39	0.83073	0.371517
X_1^2	2.30	1	2.30	0.00330	0.954663
X_2	**45613.43**	**1**	**45613.43**	**65.40048**	**0.000000**
X_2^2	**7718.17**	**1**	**7718.17**	**11.06630**	**0.002936**
X_3	2069.02	1	2069.02	2.96656	0.098425
X_3^2	**3787.91**	**1**	**3787.91**	**5.43110**	**0.028913**
Error	45613.43	23	697.45		
Total SS	7718.17	29			

Bolded values $p \leq 0.05$
Source: own elaboration.

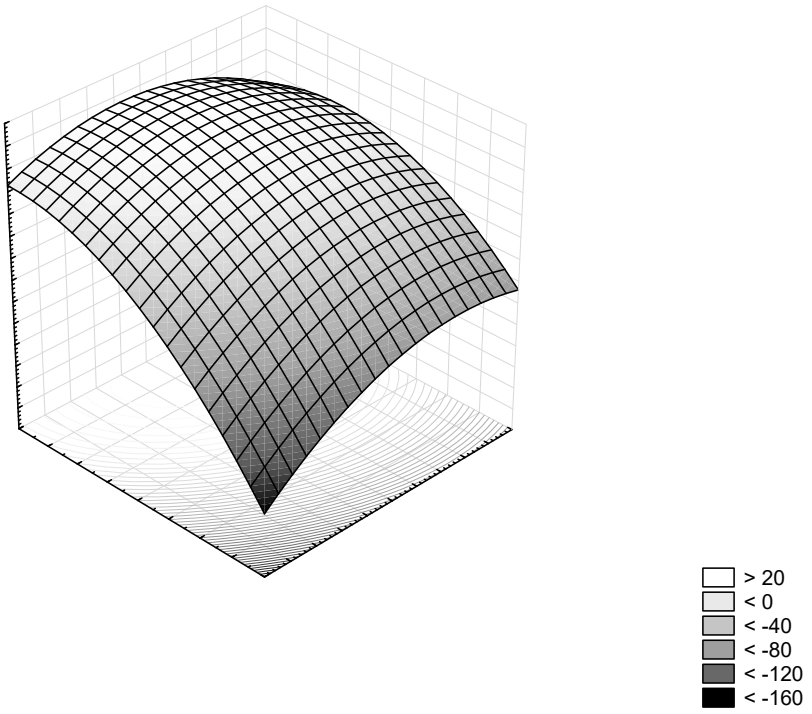
The highest decolourization, amounting to 34.57%, was achieved in the case in which 5% v/v sugar beet molasses vinasse was added to the substrate and the process was carried out at 37°C and with an initial pH value of 7.0. These results do not correspond to the highest biomass growth (16.58-fold), which was observed in case of the 25% v/v addition of BMV, temp. 37°C, pH=5.5. The highest removal of melanoidins, caramel and IDPAH amounted to approx. 50%, 6% and 35%. In any runs of the experiment, the degree of COD reduction was not greater than 14%. HPLC analysis of the vinasse after the process of decolourization confirmed that the bacterial strain used is homofermentative and can synthesize lactic acid in an amount of 10 $g \cdot dm^{-3}$. In almost all variants of the experiment, the glucose was completely assimilated.

The results created a model (R^2=0.7865) describing the dependence of the decolourization degree (Y%) of input variables, i.e. the temperature (X_2) and the vinasse concentration (X_3):

$Y=-22.9255+53.3933\ X_2+16.1645\ X_2^2+11.3242X_3^2$.

After maximizing the value obtained an objective function, set the optimum value of factors statistically significant (X_2 and X_3), which amounted to 35.8°C and 30%. Based on previous studies an initial pH of 6.5 was determined. The effect of vinasse concentration and temperature on decolourization is shown in figure 1.

Figure 1. Effect of vinasse concentration and temperature on vinasse decolourization



Source: own elaboration

The maximum colour removal in the given conditions was 26%. Most of the world's research focuses on the decolourization of cane molasses vinasse or coloured compounds derived from it. The exception is the study of Jiranuntipon et al. (2008), who attempted to decolourize cane molasses vinasse, sugar beet molasses vinasse, caramel and Viandox sauce. These substances, depending on the variant, accounted for 20%; 41.5%; 30% and 13.5% v/v of culture medium for bacteria consortium obtained from waterfall sediments (pH_0=4.0, T=30°C). The highest decolourization, amounting to 17.5%, the authors reported for a medium containing cane molasses vinasse. In the case of sugar beet molasses vinasse, the degree of reduction in colour was 8.02%. Interestingly, in the medium of caramel, neither the colour removal nor the biomass growth were

observed. In the work presented in this study the reduction of caramel - coloured component of BMV was also at the lowest level of 6% and, in the case of using a mixed culture of lactic acid bacteria there was even an increase of these compounds in the medium (data not shown). This phenomenon can be explained by the high process temperature, high acidity, high osmotic pressure and high specific gravity caramel (Belitz et al., 2004; Chung et al., 1999; Jiranuntipon al., 2008). The content of caramel in sugar beet molasses vinasse may further hinder the process of decolourization. Jiranuntipon et al. (2008) found that the initial pH value is determined by the ability of removing coloured compounds by the bacteria, and they observed the highest decolourization with an initial medium pH of 4.0. Similar conclusions were reached by Ohmomo et al. (1988) during the decolourization of melanoidins obtained from sugarcane and sugar beet molasses vinasse. The maximal decolourization of 28% was observed with *Lactobacillus hilgardii* W-NS grown under conditions of pH 4.0 or 4.5. Migo et al. (1993) reported, however, that melanoidins solubility depends on pH and it is more soluble in an alkaline pH than in an acidic pH. This statement is confirmed by Mohan et al. (2007) who optimized the process conditions of anaerobic pre-treatment and diluted to the value of the absorbance in the range of 2.8-3.0 (measured at 475 nm) sugarcane molasses vinasse. Using the bacterial consortium PAO1 *Pseudomonas aeruginosa*, *Stenotrophomonas maltophila* and *Proteus mirabilis* the authors observed the highest (67%) decolourization at neutral pH (pH=7.0). Santal et al. (2016) proposed the same pH for the process of removing the melanoidins obtained from the sugarcane molasses vinasse by the bacteria *Paracoccus pantotrophus*. Sirianuntapiboon et al. (2004) observed the highest decolourization of coloured compounds obtained from sugarcane molasses vinasse by acetogenic bacteria under a pH of 6.0. The role of pH was also emphasized by Adikane et al. (2006). During the colour removal of 12.5% v/v sugarcane molasses vinasse they found that the amount of decrease in pH during the culture determines the degree of decolourization. The highest coloured substances removal, equal to 69%, Adikane et al. (2006) was noted in an experiment in which the initial and final pH difference was the greatest. These authors for microbiological vinasse decolourization used a sample of soil collected from the vicinity of the distillery. The cited authors speculate that a high degree of colour reduction, especially in the pH range of 6-7 may be due to the improved microbial growth under these conditions. Tondee and Sirianuntapiboon (2008) and Limkhuansuwan and Chaiprasert (2010) conducted the decolourization process of isolated coloured compounds from sugarcane molasses vinasse by *L. plantarum* No. PV71-1861 and *L. plantarum* SF5.6, respectively.

Both research groups have reported a pH of 6.0 as being optimal for the process. Zuraida al. (2013) have used the bacteria *L. delbruckii* for decolourization of waste water from the textile industry. They achieved their best results under the conditions of initial pH equal to 6.0 and a temperature of 37°C.

Kumar et al. (1997) consider that the effectiveness of decolourization depends on the temperature of the process. Mohana et al. (2007) and Santal et al. (2016) in their studies found that increase of the decolourization is accompanied by an increase of the temperature used in the experiments, but only to a certain level. These authors achieved their best results at a temperature of 37°C and further increasing it resulted in a decrease in the efficiency of the process. A similar correlation was noted by Limkhuansuwan and Chaiprasert (2010) who considered a temperature of 30°C as being optimal for their experiment.

It should be noted that in the studies cited: Santal et al. (2016), Adikane et al. (2006) and Kumar et al. (1997) have decolourized much diluted vinasses, which accounted for 5%, 12.5% and 12.5% of the volume of the substrate. Santal et al. (2016) further found that increasing the concentration of the vinasse decreases the degree of decolourization, and the addition of vinasse in an amount exceeding 25% v/v inhibits the growth of bacteria. Limkhuansuwan and Chaiprasert (2010) and Tondee and Sirianuntapiboon (2008) did not carry out an experiment with distilling vinasse in their research, and only isolated coloured compounds with a specific, less than 10 kDa molecular weight. Sirianuntapiboon et al. (2004) and Ohmomo et al. (1988) also use decolourization substances obtained from sugarcane molasses vinasse so that the culture medium does not contain additional bacterial growth inhibitory substances present in the vinasse.

4. Conclusion

Both the process conditions and the concentration of vinasse in the culture medium influence the effectiveness of decolourization. The optimal pH and temperature vary depending on the strain of organism and the type of vinasse. In this work, the optimal conditions for decolourization of sugar beet molasses vinasse by the bacteria *Lactobacillus plantarum* MiLAB393 were determined: pH_0=6.5; T=35.8°C. The process for colour removal depends also on the degree of contamination and the vinasse concentration in the culture medium. Also demonstrated was the ability of the bacteria to remove colour from a substrate containing up to 30% v/v BMV. Further research should

focus on trying to replicate transfer under the optimal conditions of the process on a larger scale. The proposed process is one of the vinasse treatment steps - decolourization. After applying additional anaerobic treatment we receive waste product with reduced content of harmful coloured compounds and pollutions. Treated vinasse can already be utilized by disposal on the field or re-used, eg. for the feeding of animals. With this solution, the process of ethanol production based on vinasse will become more environmentally friendly. In summary, the use of waste sugar beet molasses from sugar production to produce ethanol and possibility to re-using vinasse which is formed after the process will be a part of some elements of sustainable development.

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Usuwanie barwy z buraczanego wywaru melasowego z wykorzystaniem metody mikrobiologicznej – wpływ parametrów procesu oraz stopnia rozcieńczenia wywaru

Streszczenie

W gorzelniach, jako produkt uboczny powstaje niebezpieczny dla środowiska wywar. Związki barwne zawarte w buraczanym wywarze melasowym (BWM) czynią go najbardziej problematycznym odpadem gorzelniczym. Tradycyjne metody usuwania ładunku zanieczyszczeń ścieków nie pozwalają na jednoczesne usunięcie barwy. W pracy przedstawiono metodę mikrobiologicznego usuwania związków barwnych zawartych w BWM. Zoptymalizowano warunki procesu (pH i temperaturę) oraz dobrano stężenie wywaru. Zastosowane bakterie *Lactobacillus plantarum* MiLAB393 w 26% usunęły barwę z BWM stanowiącego 30% v/v podłoża hodowlanego w pH_0=6,5 i 35,8°C.

***Słowa kluczowe**:* dekoloryzacja, buraczany wywar melasowy, *Lactobacillus plantarum*, bakterie fermentacji mlekowej