African Journal of Biotechnology Vol. 11(14), pp. 3464-3475, 16 Febrruary, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3029
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Decolorization of sugar beet distillery effluent using mixed cultures of bacteria of the genus *Bacillus*

Małgorzata Krzywonos

Department of Bioprocess Engineering, Wrocław University of Economics, Komandorska 118/120, 53-345 Wrocław, Poland. E-mail: malgorzata.krzywonos@ue.wroc.pl. Tel: +48 71 368 0872. Fax: +48 71 368 0753.

Accepted 3 January, 2011

The colour of sugar beet stillage is produced mainly by two groups of colorants: melanoidins (from the Maillard reaction of sugars (carbohydrates) with proteins (amino groups)), and caramels (from overheated sugars). With its high coloured matter content and high chemical oxygen demand (COD), distillery stillage cannot be treated by conventional biological methods. The aim of this study was to investigate the effect of temperature and medium supplementation on the decolorization of sugar beet stillage. Two mixed bacterial cultures of the genus *Bacillus* (C1 and C2) were tested for colour removal ability. Sugar beet stillage (W) was enriched with glucose (GLU), salts (S), and yeast extract (YE). Three temperatures were tested: 25, 35 and 45°C. Only at 25°C was a decrease in colorant content observed regardless of culture used and medium supplementation. At 25°C, the highest colorant removal was obtained with culture C2 when media W+S+YE and W+S+GLU+YE were used (34 and 35%, respectively). Melanoidins were degraded at 25 and 35°C, but not at 45°C. Moreover, at 45°C with all the media used, an increase in caramel content was observed. Spectrophotometric and high performance liquid chromatography (HPLC) analyses of treated wastewater demonstrated that the colorants underwent biotransformation.

Key words: Colorant, melanoidins, distillery wastewater, aerobic bacteria, decolorization, *Bacillus*.

INTRODUCTION

Regardless of the feedstock used (sugar cane, sugar beet molasses and sugar beet), ethanol production yields large volumes of dark brown high-strength distillery wastewater. Distillery effluents are characterized by a high content of colorants and high concentrations of organic pollutants expressed as COD (more than 100 g O_2/l) and BOD_5 (50-75 g O_2/l). Although, sugar beet molasses and sugar cane molasses stillage display a similar dark brown colour, the colorants have different origins. Those found in sugar cane molasses stillage are mainly plant pigments associated with polysaccharides, those inherent in sugar beet molasses stillage are alkaline degradation products of hexoses, melanoidins and caramels (Coca et al., 2004; Benito et al., 1997), whereas those present in sugar beet stillage are melanoidins and caramels.

Melanoidins are recognised as being acidic compounds with a charged nature. The composition of melanoidins depends on the reaction conditions, mainly temperature, heating time, pH, water content and the nature of reactants (Coca et al., 2004). Caramels are thermal degradation products of sugars. They are formed by heating concentrated sucrose syrups at temperatures above 210°C and pH from 3 to 9. Caramelization occurs in ethanol production when beer is heated strongly, eample, during distillation. However, the presence of impurities in low concentration, mainly iron, reduces the caramelization temperature by up to 40°C. Alkaline degradation reactions take place in a beet sugar factory at pH from 8 to 11 mainly during the purification step where the temperature rises up to 85°C and the pH increases to basic values (11 to 12) (Coca et al., 2004). However, during ethanol production from sugar beet, distillation takes place at high temperatures but at acidic pH values. These conditions are not suitable for alkaline degradation reactions.

Colorants have antioxidant properties; they are toxic to many microorganisms involved in conventional treatment

processes, which are not efficient in colorant degradation. In conventional anaerobic-aerobic processes colorants are removed with an efficiency of approximately 7% (Pena et al., 2003). In the activated sludge process, high-molecular-weight coloured substances are degraded only to low-molecular-weight compounds. It has been further observed that if colorants are not removed, the pigments will still be present in the stillage, which indicates that the COD value will remain at the initial level (Mane et al., 2006). Attempts have been reported to remove pigments from distillery effluents by physicochemical methods such as adsorption (Mane et al., 2006), coagulation-flocculation (Zayas et al., 2007), oxidation processes involving Fenton reactions (Mohana et al., 2009), ozonation (Pena et al., 2003), electrochemical oxidation using various electrodes and electrolytes (Manisankar et al., 2004), or nanofiltration and reverse osmosis (Mohana et al., 2009). Efforts have also been reported to combine the physicochemical methods mentioned above (Chaudhari et al., 2007; Zayas et al., 2007). Physico-chemical methods are generally applied as a stage that follows the anaerobic treatment process. However, because of the high operating costs involved, as well as the fact that large sludge quantities are produced as a result, physicochemical methods are not used on a commercial scale.

Several literatures contain references to biological methods for the decolorization of distillery effluents (Pant and Adholeya, 2007a). It is essential to note, however, that the experiments reported there pertain primarily to pigment removal from diluted cane molasses stillage after anaerobic treatment, and also to the removal of synthetic pigments. The cultures used in the reported studies contained cyanobacteria (Kalavathi et al., 2001); *Pseudomonas fluorescens* (Mohana et al., 2007); *Aspergillus* (Shayegan et al., 2005); *Phanerochaete chrysosporium* (Kumar et al., 1998); *Penicillium decumbens* (Jimenez et al., 2005); *Penicillium pinophilum, Alternaria gaisen, Aspergillus niger*, *Aspergillus flavus* (Pant and Adholeya, 2007b,c); *Geotrichum candidum* (Kim and Shoda, 1999); *Trametes versicolor* (Kumar et al., 1998); *Bacillus* sp. (Bharagava et al., 2009; Chaturvedi et al., 2006; Kaushik and Thakur, 2009; Nakajima-Kambe et al., 1999; Kumar and Chandra, 2006), and *Lactobacillus* (Tondee and Sirianuntapiboon, 2008). No references to biological decolorization of sugar beet stillage have been found in the literature.

However, the successful decolorization of sugar cane molasses stillage using mixed cultures of the genus *Bacillus* (Bharagava et al., 2009) prompted us to use mixed bacterial cultures of *Bacillus* sp. to decolorize sugar beet stillage. The novelty of the research reported here lies in the decolorization process of the sugar beet stillage and its usage in a non-diluted form, and also in the use not of synthetic pigments, but of those present in distillery stillage. The aim of the study was to determine the influence of temperature and medium supplementation on the decolorization process of the sugar beet distillery effluent.

MATERIALS AND METHODS

Medium

Samples of distillery wastewater were obtained from a distillery plant (Lower Silesia, Poland) where ethanol was produced from sugar beet. Before use, solid particles were removed from the stillage *via* filtration through filter paper. The liquid phase obtained after separation was stored at -20°C before incorporation into the growth media. The pH and density of the stillage were 5.25 and 5.5, respectively. The composition of the liquid phase was as follows (g/L): Chemical oxygen demand (COD), 48.3; total organic carbon (TOC), 11.7; total nitrogen (TN), 1.512; ammonia nitrogen ($N\text{-}NH_4$), 0.154; total phosphorus (TP), 0.29; phosphate phosphorus ($P\text{-}PO_4$), 0.199; reducing substances determined before hydrolysis, 5.38; reducing substances determined after hydrolysis, 14.29; glycerol, 2.98; glucose, 0.17; lactic acid, 2.37; propionic acid, 1.318; acetic acid, 1.92; malic acid, 0.051; pyroglutamic acid, 0.81; succinic acid, 0.14; isobutyric acid, 0.9; caramels, 5.78 and melanoidins, 3.73.

Sugar beet stillage (W) used for decolorization was enriched with glucose (GLU; 10 g/L; POCH S.A., Gliwice, Poland), salts (S; 5 g/L K_2HPO_4, and 0.75 g/L $MgSO_4.7H_2O$, POCH S.A., Gliwice, Poland), and yeast extract (YE; 5 g/L, BTL sp. Z O. O., Lodz, Poland). Glucose was added separately after sterilization of the stillage and additives at 121°C for 15 min. The pH was adjusted to 7.0 with 2 M NaOH. The medium used for the decolorization in four variants consisted of the sugar beet stillage (W) supplemented with salts (W+S), with salts and glucose (W+S+GLU), with salts and yeast extract (W+S+YE), and with salts, yeast extract and glucose (W+S+YE+GLU).

Microorganisms

In the studies reported herein, two mixed bacterial cultures of the genus *Bacillus* were used. One of these (referred to as C1) consisted of seven strains of the genus *Bacillus*: two strains of *B. circulans*, and single strains of *B. laterosporus, B. filicolonicus, B. stearothermophilus, B. acidocaldarius* and *B. licheniformis* (Cibis et al., 2004). The other culture (referred to as C2) consisted of 21 strains of thermo- and mesophilic *Bacillus* spp.: *B. circulans* (2 strains), *B. laterosporus, B. filicolonicus, B. stearothermophilus* (2 strains), *B. acidocaldarius* and *B. licheniformis*, *B. smithii* (2 strains), *B. sphaericus* (2 strains), *B. licheniformis* (3 strains), *B. subtilis* (2 strains), *B. mycoides, B. coagulans, B. megaterium,* and *B. polymyxa.* Culture C1 was previously used for biodegradation of potato stillage (Krzywonos et al., 2008). Culture C2 was obtained by the enrichment of C1 with mesophilic and thermophilic bacteria from the collection of the microorganisms belonging to the Department of Biotechnology and Food Microbiology (Wroclaw University of Environmental and Life Sciences), the Institute of Fermentation Technology and Microbiology (Technical University of Lodz), and the German Collection of Microorganisms and Cell Cultures Ltd. (Brunswick). Both mixed cultures used in the study were kept frozen in a 20% (w/v) glycerol solution.

Inoculum preparation

The preparation of inoculum commenced with the transfer of the frozen microorganisms (0.1 ml) to a 100 ml flask containing 50 ml of nutrient broth under aseptic conditions. The flask was incubated at 37°C for 24 h before use in the experiments. The volume of the

inoculum for the decolorization experiments comprised 1 ml of bacteria grown in nutrient broth.

Process conditions

Decolorization of the nutrient-supplemented sugar beet stillage by the mixed cultures was carried out for 7 days in 300 ml flasks (each containing 100 ml of the medium) under the following conditions: temperatures of 25, 35 and 45°C; agitation of 150 rpm. Samples were collected every 24 h. All experiments were conducted aseptically in triplicate. Average values are reported.

Analytical techniques

The medium was centrifuged at 9,000 g (Sigma® 4K15) for 15 min, and suspended solids (SS) were determined gravimetrically by drying at 50°C for 24 h and then at 105°C until a constant weight was obtained. 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 assayed spectrophotometrically using Dr. Lange cuvette tests (Anon, 2000). Ammonia nitrogen ($N\text{-}NH_4$) concentration was measured by distillation with water vapour in the Parnas apparatus. Total nitrogen (TN) was determined by the Kjeldahl method. Glucose, glycerol and organic acid concentrations (lactic, acetic, propionic, pyroglutamic, succinic, malic, and isobutyric acids) were determined by HPLC (Knauer; detectors UV-VIS and RI; 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, 40°C).

Decolorization yield

After centrifugation, the supernatant was diluted with 0.9% NaCl, and the diluted solution was analyzed for colour intensity at 475 nm with a UV-Visible spectrophotometer. Decolorization activity was expressed as the difference between initial and final absorbance divided by initial absorbance. The concentrations of melanoidins and caramels were measured spectrophotometrically (at 282 and 300 nm) and then calculated (Sapronov, 1963). 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, 27°C). The detection wavelength was set at 290 nm (Bharagava et al., 2009).

Data analysis

Microsoft Excel 2000 was used for statistical analysis of the data with the level of significance set at 95%. One-way analysis of variance (ANOVA) was applied to assess statistical differences between the results. Differences were considered as significant at $p \leq 0.05$.

RESULTS

Three temperatures were tested: 25, 35 and 45°C. Only at 25°C was a significant decrease ($p \leq 0.05$) in colorant content observed regardless of culture used and medium supplementation (Figure 1). At 25°C, the highest significant colorant removal, 34 and 35%, was obtained ($p \leq 0.05$) with media W+S+YE and W+S+GLU+YE, respectively and culture C2; the lowest significant colorant removal (9%) being achieved with culture C1 when the process was performed on the W+S medium. With culture C2 on the same medium, colour removal was twice as high (18%) ($p \leq 0.05$) (Figure 1).

At 35°C with culture C1, an increase in colorant content was observed in all media ($p \leq 0.05$) (Figure 1); with culture C2, the content of colorants increased only in the W+S medium. With the C2 consortium and the other three media used, while colorant content decreased (Figure 1). In general, however, the efficiency of colour removal at 35°C was lower than in any of the experiments performed at 25°C and did not exceed 11%. While at 45°C, a decline in decolorization activity was observed in all the experiments, and colorant content increased significantly ($p \leq 0.05$) (from 20 to 42%) (Figure 1). With both cultures, the largest increase was found to occur in the stillage supplemented with salts and YE ($p > 0.05$). The effect of temperature on colorant removal from sugar beet stillage is evident. When the temperature rose, the efficiency of decolorization decreased (Figure 1).

In this study, COD was chosen as the measure of colorant degradation. When the experiments were performed at 25°C with culture C1, the highest COD removal, which totalled 4%, was observed in the stillage medium supplemented with salts and glucose ($p \leq 0.05$). With culture C2, removal of COD amounted to 8% in all but one of the media. The one exception was in the W+S+YE+GLU medium, where COD content increased slightly (Figure 2). Furthermore, when higher temperatures were employed, COD removal increased ($p \leq 0.05$), ranging from 13 to 54%, and from 3 to 35% at 35 and 45°C, respectively. Yet there was one exception at 45°C (with culture C1 on W+S+GLU), where COD content slightly increased (Figure 2). The results obtained at 25°C revealed that when decolorization occurred, COD removal was low or almost negligible ($p \leq 0.05$) (Figures 1 and 2). The increase in COD content and its low removal might be associated with synthesis of organic acids in higher amounts (Tables 1 to 3). This effect was observed predominantly with culture C1 at 25°C (Table 1).

Meanwhile, melanoidins were degraded at 25 and 35°C, but at 45°C their content increased in all media (Figure 3). With culture C2, significantly higher melanoidin removal was observed at 25 than 35°C for all media tested ($p \leq 0.05$). This was also the case with C1 and the W+S+YE medium. In the other media, culture C1 provided higher melanoidin removal at 35°C than 25°C ($p \leq 0.05$). On the other hand, caramel content increased in all the media at 45°C, regardless of the culture used (Figure 4). However, at 25 and 35°C, with culture C2 grown in media W+S, W+S+YE and W+S+GLU, caramel content decreased, varying between 7.7 and 11.4%. With culture C1, the effect was more complex: in medium W+S+YE at 25 and 35°C and medium W+S at 35°C, there was a decrease in caramel content, which ranged

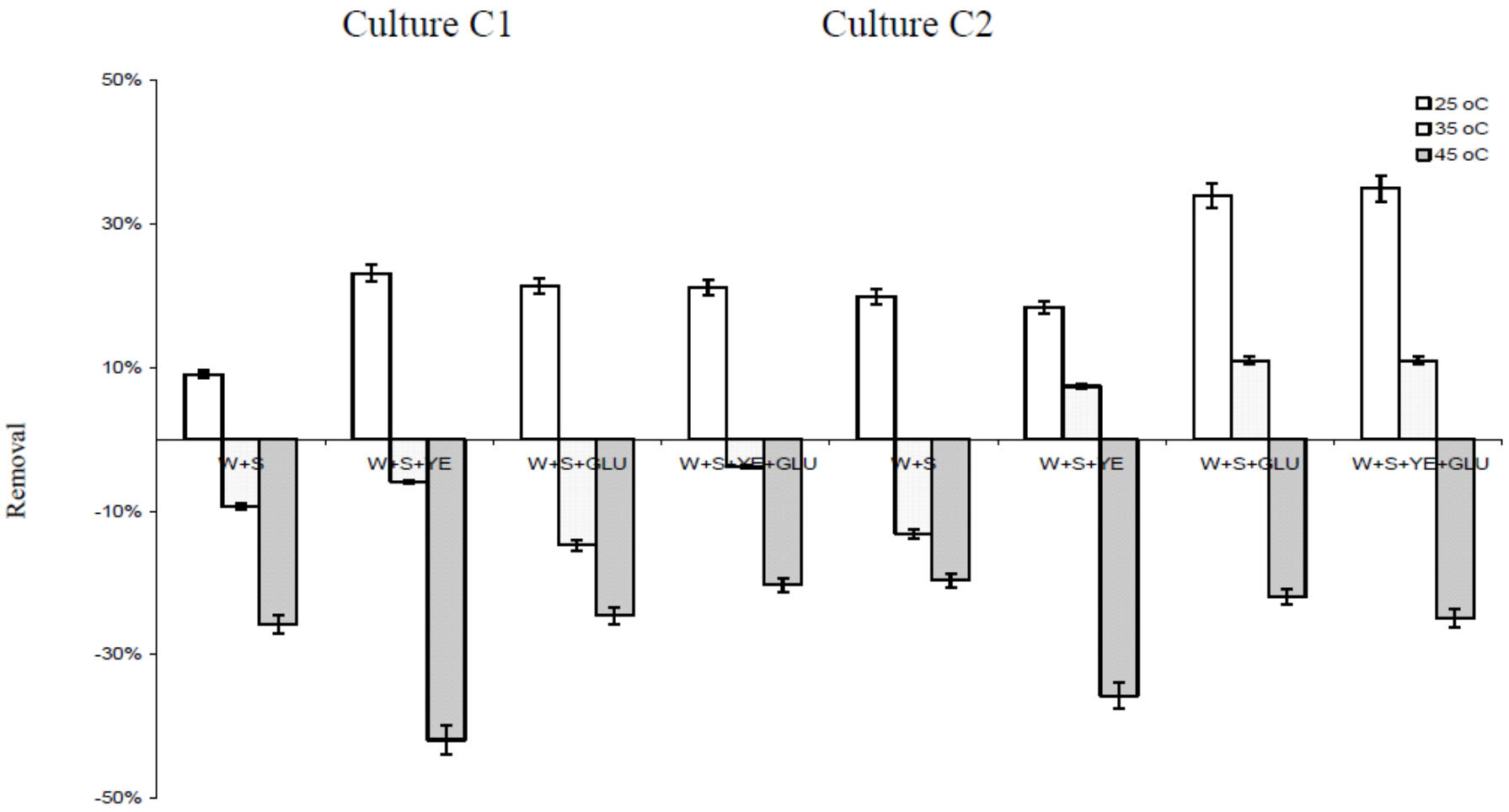

Figure 1. Colorants removal during the experiments performed at 25, 35 and 45°C. Letters above the histogram bars represents analysis of variance (ANOVA). Pairs with values not statistically different (p> 0.05) are marked with superscript letter.

between 4.1 and 22.8%; otherwise it increased.

High performance liquid chromatography (HPLC) analyses were performed to confirm degradation of the colorants present in the stillage. At 25°C in all the media, new peaks appeared in the HPLC chromatogram compared with the control (Figure 5). Quantification was made by calculating the peak areas at 290 nm. Results obtained at 25°C with both cultures are summarized in Table 4. With culture C2, the peak area (W+S+YE and W+S+YE+GLU media) was approximately the same as that for the control, indicating little or no colorant degradation. The highest decrease in the peak area was obtained with both cultures for the W+S+GLU medium.

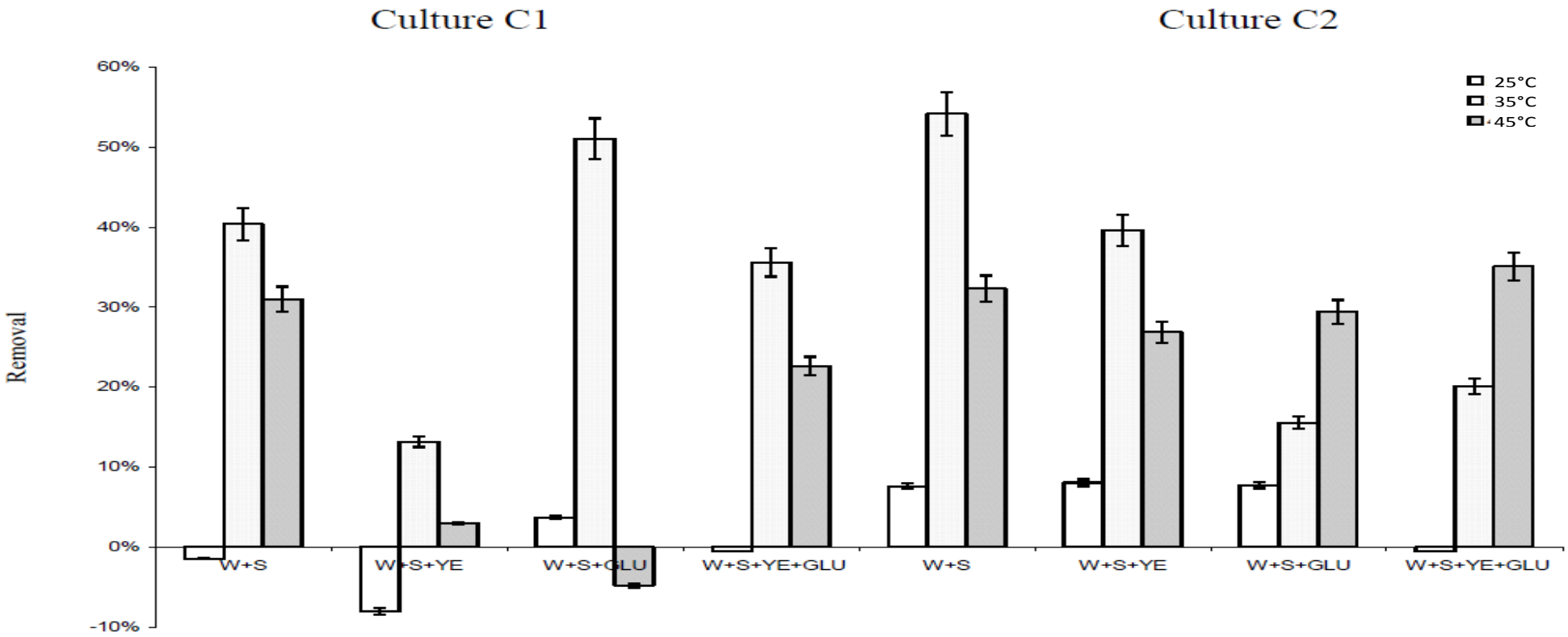

Figure 2. Chemical oxygen demand (COD) removal during the experiments performed at 25, 35 and 45°C. All values are statistically different (p≤ 0.05).

Table 1. Glucose, glycerol and organic acids removal during the experiments performed at 25°C.

Removal (%)	Culture C1				Culture C2			
	W+S	W+S+YE	W+S+GLU	W+S+ GLU+YE	W+S	W+S+YE	W+S+GLU	W+S+GLU+YE
Glucose	100 ± 0	32.10 ± 0.69	82.6 ± 0.79	59.53 ± 0.68	100 ± 0	81.84 ± 0.69	79.9 ± 0.81	100 ± 0
Glycerol	8.48 ± 0.36	4.85 ± 0.36	11.5 ± 0.58	98.45 ± 0.95	100 ± 0	24.19 ± 0.41	85.3 ± 0.74	75.23 ± 0.48
Citric acid	100 ± 0	-352.73 ± 22.89	100 ± 0	75.50 ± 0.92	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Lactic acid	-78.98 ± 0.45	-445.43 ± 45.26	-759.3 ± 36.25	-495.50 ± 23.25	47.23 ± 0.65	43.72 ± 0.56	-1212.0 ± 54.48	-1480.00 ± 35,25
Acetic acid	100 ± 0	-96.06 ± 0.95	59.5 ± 0.89	12.66 ± 0.67	3.70 ± 0.25	40.88 ± 0.25	68.3 ± 0.47	78.68 ± 0.87
Pyroglutamic acid	-85.46 ± 0.79	-59.73 ± 0.48	-202.3 ± 2.69	-95.54 ± 0.87	35.74 ± 0.58	46.54 ± 0.57	51.6 ± 0.78	31.53 ± 0.48

‘‘-’’ before the number denotes an increase in value. All values are statistically different (p≤ 0.05).

Table 2. Glucose, glycerol and organic acids removal during the experiments performed at 35°C.

Removal (%)	Culture C1				Culture C2			
	W+S	W+S+YE	W+S+GLU	W+S+GLU+YE	W+S	W+S+YE	W+S+GLU	W+S+GLU+YE
Glucose	100 ± 0	4.15 ± 0.25	100 ± 0	93.95 ± 0.85[a]	61.78 ± 0.87	13.85 ± 0.45	96.5 ± 0.95	94.64 ± 0.85[a]
Glycerol	100 ± 0	86.15 ± 0.73	100 ± 0	100 ± 0	100 ± 0	100 ± 0	65.2 ± 0.73	69.89 ± 0.84
Citric acid	100 ± 0	100 ± 0	100 ± 0	100 ± 0	50.47 ± 0.56	56.93 ± 0.87	100 ± 0	100 ± 0
Lactic acid	71.72 ± 0.82	94.55 ± 0.89	82.4 ± 0.96	75.39 ± 0.92	74.14 ± 0.86	100 ± 0.00	85.9 ± 0.91	37.30 ± 0.59
Acetic acid	68.38 ± 0.54	77.49 ± 0.68	11.2 ± 0.25	58.12 ± 0.65	100 ± 0	41.44 ± 0.58	-6.0 ± 0.45	-6.03 ± 0.32
Pyroglutamic acid	47.48 ± 0.74	39.26 ± 0.87	-18.6 ± 0.43	68.34 ± 0.59	55.35 ± 0.74	-504.78 ± 34.2	-10.0 ± 0.24	-77.93 ± 0.65
Isobutyric acid	100 ± 0	-80.90 ± 0.97	100 ± 0	-589.80 ± 15.48	100 ± 0	60.22 ± 0.73	100 ± 0	-152.79 ± 2.56

''-'' before the number denotes an increase in value. Pairs with values not statistically different (p> 0.05) are marked with superscript letter.

Table 3. Glucose, glycerol and organic acids removal during the experiments performed at 45°C.

Removal (%)	Culture C1				Culture C2			
	W+S	W+S+YE	W+S+GLU	W+S+GLU+YE	W+S	W+S+YE	W+S+GLU	W+S+GLU+YE
Glucose	100 ± 0	100 ± 0	96.0 ± 0.98[a]	82.22 ± 0.92	100 ± 0	100 ± 0	97.9 ± 0.94[a]	94.83 ± 0.98
Glycerol	100 ± 0	0.1 ± 0.05	0.4 ± 0.15	9.55 ± 0.38	100 ± 0	100 ± 0.00	100 ± 0	100 ± 0
Citric acid	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Lactic acid	47.25 ± 0.56	-11.90 ± 0.68	-270.6 ± 5.12	-231.43 ± 4.87	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Acetic acid	-178.25 ± 4.36	-54.49 ± 0.94	-136.3 ± 3.48	-131.68 ± 2.79	-78.50 ± 0.89	-46.13 ± 0.81	-96.1 ± 0.87	-45.64 ± 0.84
Pyroglutamic acid	49.42 ± 0.63	-117.44 ± 1.59	-89.1 ± 0.84	80.23 ± 0.91	100 ± 0	-59.79 ± 0.88	16.6 ± 0.56	-512.10 ± 14.78
Isobutyric acid	100 ± 0	100 ± 0	100 ± 0	39.67 ± 0.59	18.87 ± 0.65	6.78 ± 0.54	20.2 ± 0.62	46.24 ± 0.79

''-'' before the number denotes an increase in value. Pairs with values not statistically different (p> 0.05) are marked with superscript letter.

With culture C1, the highest decrease in the peak area (17.08%) was observed when the stillage was supplemented with salts and glucose (W+S+GLU). However, when the salt-medium was enriched with YE, the C1 culture produced an increase in the peak area of 23.86%. To compare the efficiency of decolorization, the height of the peak with a retention time of 5.07 min was used for the control and for samples taken after 7 days of degradation at 25°C. Only in two experiments was the height reduced by less than 59%. With the W+S+YE medium, the decrease in height was the lowest, regardless of the culture. The highest value was attained with both cultures when the medium was enriched with salts and glucose

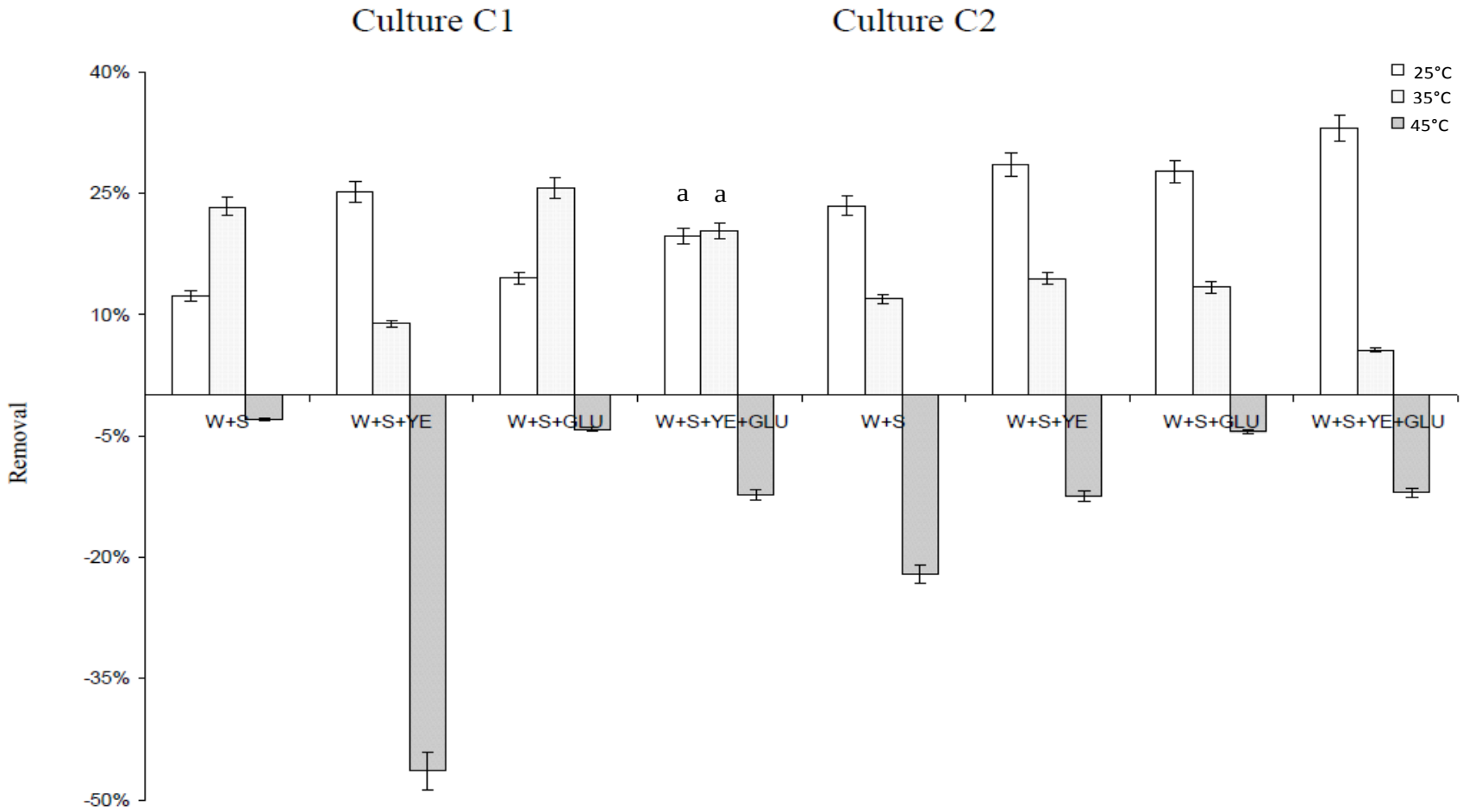

Figure 3. Melanoidins removal during the experiments performed at 25, 35 and 45°C. Letter above the histogram bars represents Analysis of Variance (ANOVA). Pairs with values not statistically different (p> 0.05) are marked with superscript letter.

(Table 4).

DISCUSSION

Decolorization efficiencies obtained in the present study are lower than those attained by Kaushik and Thakur (2009) (85% removal of colorant) with bacteria of the genus *Bacillus*, and by Bharagava et al. (2009) (70% removal) with a mixed culture containing *B. licheniformis, Bacillus sp.* and *Alcaligenes*. When Kumar and Chandra (2006) used three *Bacillus* strains (*B. brevis, B. thuringiensis* and *Bacillus* sp.) for decolorization of synthetic melanoidins, the removal of colorants approached 50%. It is worth knowing, however, that in the present study non-diluted distillery stillage with a higher initial content of colorants than in the studies by Bharagava et al. (2009), Kumar and Chandra (2006) and Kaushik and Thakur (2009) was used. Nevertheless, this study

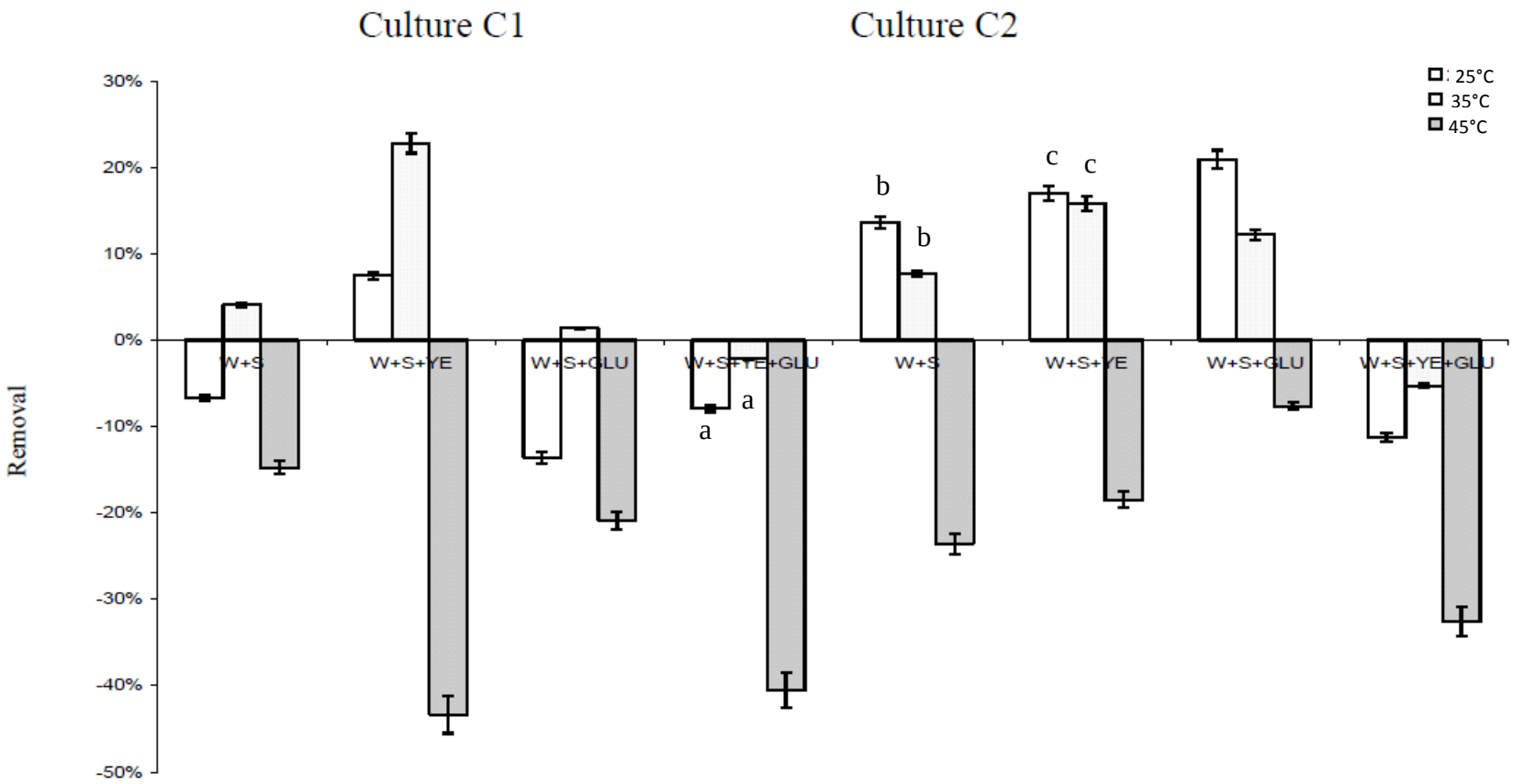

Figure 4. Caramels removal during the experiments performed at 25, 35 and 45°C. Letter above the histogram bars represents analysis of Variance (ANOVA). Pairs with values not statistically different (p> 0.05) are marked with superscript letter.

yielded results similar to those obtained by Pant and Adholeya (2009), who used different fungal isolates to extract enzymes for the decolorization of a non-diluted distillery effluent. The highest colour removal (37%) was achieved with the extract from *Pleurotus florida* EM1303. However, the non-diluted distillery effluent used by Pant and Adholeya (2009) underwent hydroponic pretreatment which aimed at reducing the high nitrogen content. Compared with this present study, the reduction in colour and COD (61.5 and 65.4%, respectively) achieved by Pant and Adholeya (2010) was higher, but they treated a post-anaerobically digested distillery effluent with a mixed culture of fungi.

A decline in decolorization activity with the increase in incubation temperature (from 37 to 45°C) was observed by Junnarkar et al. (2006), when they used a novel bacterial consortium which was selected based on rapid decolorization of Direct Red 81. This temperature-dependent decline can be attributed either to the loss of the cells' viability or to the denaturation of the cells' enzymes (Gomare et al., 2009; Pearce et al., 2003). Such behaviour can be explained as suggested by Wong and Yuen (1996), who postulate that degradation products formed and

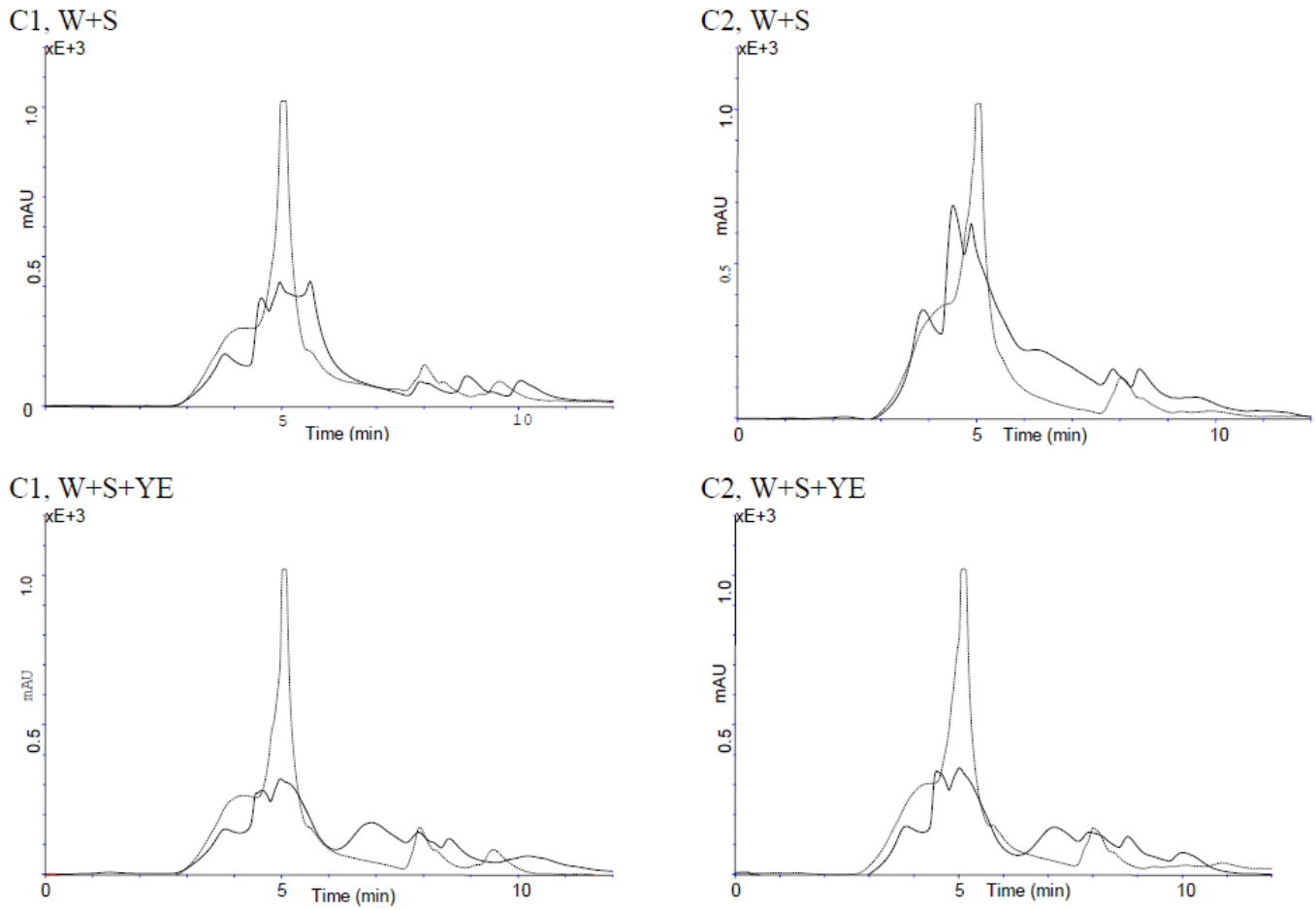

Figure 5. Chromatograms of colorants before (dotted line) and after (solid line) 7 days of decolorization (25°C).

saturated in the culture medium may affect cell viability and decolorization activity. The decrease in decolorization activity at 35 and 45°C might be due to the changes in pH during the process, which inhibited microbial growth and colorant degradation. This finding is consistent with the results obtained by Jiranuntipon et al. (2008), who studied decolorization of synthetic melanoidins-containing wastewater by a bacterial consortium. They also suggested that the increase in colour may be due to the polymerization of melanoidins and to the toxicity of metabolites that formed and accumulated during decolorization, thereby repressing decolorization activity. According to Jiranuntipon et al. (2008), it is also possible that the absence of some nutrients markedly affected the efficiency of decolorization by the bacterial consortium. In addition, Strong (2010), who used fungi for remediation of Amarula distillery wastewater, suggested that the increase in colour was caused by various factors: the transformation of pigments to more colour-rich compounds, the increase in pH, or the combination of both the factors.

The increase in COD content and its low removal might be associated with the synthesis of organic acids in higher amounts (Tables 1 to 3). Synthesis of carboxylic acids during aerobic meso- and thermophilic stillage biodegradation was also observed when potato and wheat stillage were biodegraded with a mixed culture of bacteria of the genus *Bacillus* (Krzywonos et al., 2010; Krzywonos et al., 2009). Ohmomo et al. (1988) suggested that degradation of melanoidins is concomitant with synthesis of lactic acid. This suggestion holds true for the study reported here, especially for the experiments at 25°C (Table 1).

A reduction in the peak areas of degraded samples compared with the control sample was observed by Bharagava et al. (2009). They implied that melanoidin degradation involves the production of extracellular hydrogen peroxide and peroxidases. Peroxidase activity requires hydrogen peroxide, which is produced during glucose oxidation, and thus necessitates addition of glucose as an extra carbon source. In the work reported here, supplementation of a readily available carbon source (glucose) seems to aid the degradation of colorants at 25°C, except for W+S+YE+GLU with C2 (Table 4). This might also be an indication that decolorization occurred as a result of a reaction induced

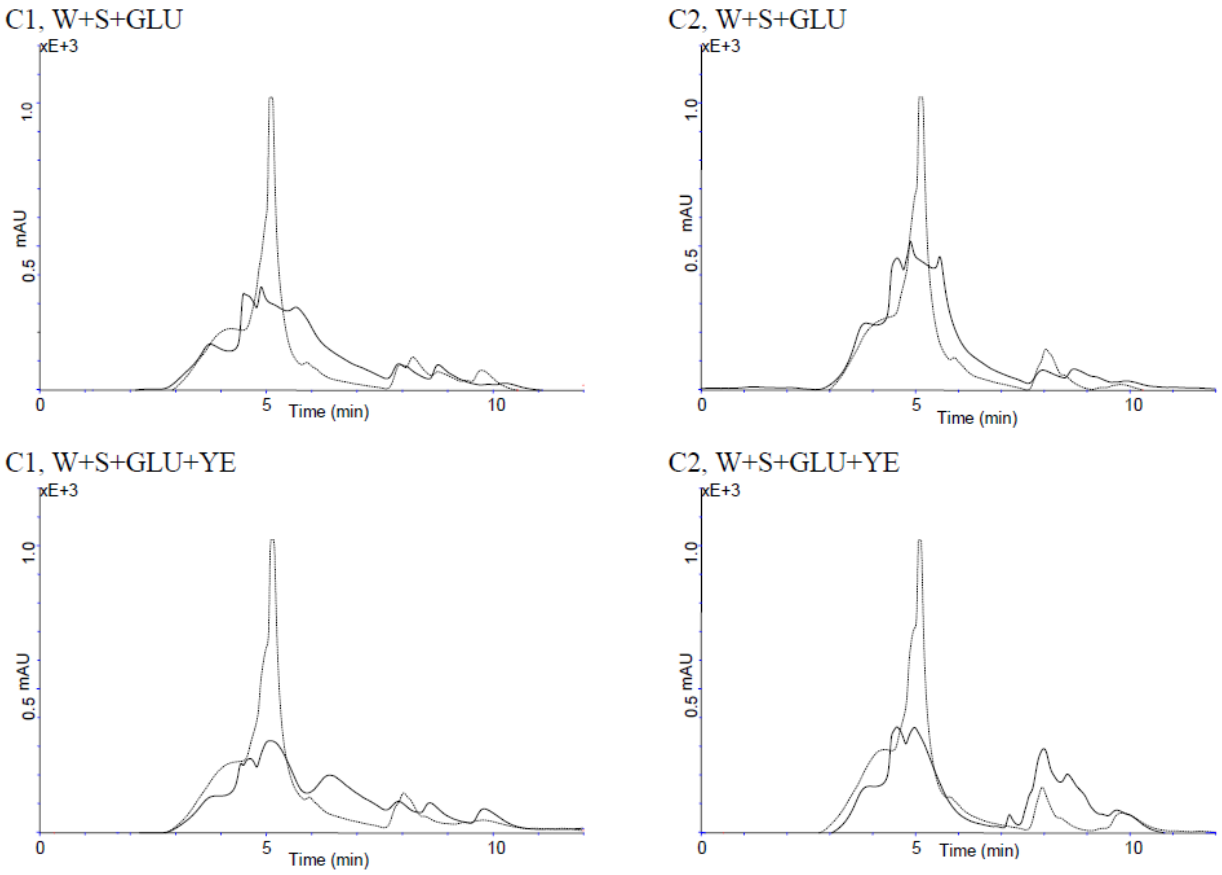

Figure 5. Contd.

by a secondary metabolite. Reduction in the content of melanoidins may be attributed to their bacterial degradation in the presence of supplementary carbon and nitrogen sources through co-metabolism (Kumar and Chandra, 2006). Chavan et al. (2006) suggest that the decline in optical density at 475 nm and the appearance of new peaks in the chromatograms indicate the occurrence of degradation. Bharagava et al. (2009) observed smaller peaks (reduced height) in the chromatogram as compared with the control samples, and postulated that the decline in colour intensity might be largely attributed to bacterial degradation of melanoidins. Bharagava et al. (2009) also implied that the formation of additional peaks might indicate formation of metabolites in the medium, as a result of biodegradation and biotransformation of melanoidins. In general, colour degradation coincides with changes in molecular structure, but total mineralization of organic matter does not seem to occur (Dwyer et al., 2008).

Conclusion

This study revealed that the bacterial consortia used were efficient only at 25°C regardless of medium supplementation. The highest colorant removal was obtained with culture C2 when W+S+YE and W+S+GLU+YE media were used (34 and 35%, respectively). Melanoidins were degraded at 25 and 35°C, but not at 45°C. At 45°C, with all the media used, an increase in caramel content was observed. Spectrophotometric and HPLC analyses of sugar beet stillage suggested that the colorants underwent biotransformation.

Microbial decolorization of distillery stillage therefore shows great promise as a cost-effective, environmentally safe biotechnology for the treatment of high-strength industrial wastewater. Future research will address the issue of supplementing different carbon sources and optimizing the amounts added. Carbon sources may be obtained for example by addition of municipal sewage, which is rich not only in carbon, but also in phosphorus and ammonia.

ACKNOWLEDGEMENTS

This study was financed by the National Science Centre

Table 4. HPLC profile of colorants degradation study at 25°C.

Parameter	Culture C1				Culture C2			
	W+S	W+S+YE	W+S+GLU	W+S+YE+GLU	W+S	W+S+YE	W+S+GLU	W+S+YE+GLU
Height reduction (%)	59.56 ± 0.3	33.12 ± 0.1	69.66 ± 0.4[a]	64.53 ± 1	65.44 ± 3	50.57 ± 0.1	68.99 ± 1[a]	62.55 ± 0.3
Area reduction (%)	13.26 ± 3[b]	-23.86 ± 5	17.08 ± 3	10.24 ± 2	6.72 ± 4[b]	0.30 ± 0.2	10.03 ± 3	-0.45 ± 0.5

"-" before the number denotes an increase in value. Reductions in height were calculated for peaks with a retention time of 5.07 min. Pairs with values not statistically different (p> 0.05) are marked with superscript letter.

(Poland) under Project no. N N312 421940. Dr C.A. Kent (School of Chemical Engineering, University of Birmingham, UK) is acknowledged for his critical reading of the manuscript.

REFERENCES

Anon (2000). Handbook of Photometrical Operation Analysis. Dr.Lange BDB 079 (Februar 2000): (In German).

Benito GG, Miranda MP, de los Santos DR (1997). Decolorization of wastewater from an alcoholic fermentation process with Trametes versicolor. Bioresour. Technol. 61(1): 33-37.

Bharagava RN, Chandra R, Rai V (2009). Isolation and characterization of aerobic bacteria capable of the degradation of synthetic and natural melanoidins from distillery effluent. World J. Microbiol. Biotechnol. 25(5): 737-744.

Chaturvedi S, Chandra R, Rai V (2006). Isolation and characterization of Phragmites australis (L.) rhizosphere bacteria from contaminated site for bioremediation of colored distillery effluent. Ecol. Eng. 27(3): 202-207.

Chaudhari PK, Mishra IM, SC (2007). Decolourization and removal of chemical oxygen demand (COD) with energy recovery: Treatment of biodigester effluent of a molasses-based alcohol distillery using inorganic coagulants. Colloids Surf. A 296(1-3): 238-247.

Chavan MN, Kulkarni MV, Zope VP, Mahulikar PP (2006). Microbial degradation of melanoidins in distillery spent wash by an indigenous isolate. Indian J. Biotechnol. 5: 416-421.

Cibis E, Krzywonos M, Trojanowska K, Miśkiewicz T, Ryznar A (2004). Biodegradation of potato slops with a mixed population of bacteria of the genus Bacillus-determination of the process conditions. EJPAU 7 (2):http://www.ejpau.media.pl/series/volume7/issue2/food/art-01.html

Coca M, García MT, González G, Peña M, García JA (2004). Study of coloured components formed in sugar beet processing. Food Chem. 86(3): 421-433.

Dwyer J, Kavanagh L, Lant P (2008). The degradation of dissolved organic nitrogen associated with melanoidin using a UV/H2O2 AOP. Chemosphere, 71(9): 1745-1753.

Gomare SS, Tamboli DP, Kagalkar AN, Govindwar SP (2009). Eco-friendly biodegradation of a reactive textile dye Golden Yellow HER by Brevibacillus laterosporus MTCC 2298. Int. Biodeterior. Biodegradation, 63(5): 582-586.

Jimenez AM, Borja R, Martın A, Raposo F (2005). Mathematical modelling of aerobic degradation of vinasses with Penicillium decumbens. Proc. Biochem. 40: 2805-2811.

Jiranuntipon S, Chareonpornwattana S, Damronglerd S, Albasi C, Delia M-L (2008). Decolorization of synthetic melanoidins-containing wastewater by a bacterial consortium. J. Ind. Microbiol. Biotechnol. 35: 1313-1321.

Junnarkar N, Murty D, Bhatt N, Madamwar D (2006). Decolorization of diazo dye Direct Red 81 by a novel bacterial consortium. World J. Microbiol. Biotechnol. 22(2): 163-168.

Kalavathi DF, Uma L, Subramanian G (2001). Degradation and metabolization of the pigment- melanoidin in a distillery effluent by the marine cyanobacterium Oscillatoria boryana BDU 92181. Enzyme Microb.Technol. 29(4-5): 246-251.

Kaushik G, Thakur IS (2009). Isolation and characterization of distillery spent wash color reducing bacteria and process optimization by Taguchi approach. Int. Biodeterior. Biodegradation, 63: 420-426.

Kim SJ, Shoda M (1999). Batch decolorization of molasses by suspended and immobilized fungus of Geotrichum Candidum Dec 1. J. Biosci. Bioeng. 88(5): 586-589.

Krzywonos M, Cibis E, Lasik M, Nowak J, Miśkiewicz T (2009). Thermo- and mesophilic aerobic batch biodegradation of high-strength distillery wastewater (potato stillage) - utilisation of main carbon sources. Bioresour. Technol. 100(9): 2507-2514.

Krzywonos M, Cibis E, Miśkiewicz T, Kent CA (2008). Effect of temperature on the efficiency of the thermo- and mesophilic aerobic batch biodegradation of high strength distillery wastewater (potato stillage). Bioresour. Technol. 99: 7816-7824.

Krzywonos M, Cibis E, Ryznar-Luty A, Miskiewicz T, Borowiak D (2010) .Aerobic biodegradation of wheat stillage (distillery wastewater) at an elevated temperature - Effect of solids separation. Biochem. Eng. J. 49 (1): 1-6.

Kumar P, Chandra R (2006). Decolourisation and detoxification of synthetic molasses melanoidins by individual and mixed cultures of *Bacillus* spp. Bioresour. Technol. 97(16): 2096-2102.

Kumar V, Wati L, Nigam P, Banat IM, Yadav BS, Singh D, Marchant R (1998). Decolorization and biodegradation of anaerobically digested sugarcane molasses spentwash effluent from biomethanation plants by white-rot fungi. Proc. Biochem. 33(1): 83-88.

Mane JD, Modi S, Nagawade S, Phadnis SP, Bhandari VM (2006). Treatment of spentwash using chemically modified bagasse and colour removal studies. Bioresour. Technol. 97(14): 1752-1755.

Manisankar P, Rani C, Viswanathan S (2004). Effect of halides in the electrochemical treatment of distillery effluent. Chemosphere, 57(8): 961-966.

Mohana S, Acharya BK, Madamwar D (2009). Distillery spent wash: treatment technologies and potential applications. J. Hazard. Mater. 163(1): 12-25.

Mohana S, Desai C, Madamwar D (2007) .Biodegradation and decolourization of anaerobically treated distillery spent wash by a novel bacterial consortium. Bioresour. Technol. 98: 333-339.

Nakajima-Kambe T, Shimomura M, Nomura N, Chanpornpong T, Nakahara T (1999). Decolorization of molasses wastewater by *Bacillus* sp. under thermophilic and anaerobic conditions. J. Biosci. Bioeng. 87(1): 119-121.

Ohomomo S, Daengsubha W, Yoshikawa H, Yui M, Nozaki K, Nakajima T, Nakamura I (1988). Screening of anaerobic bacteria with the ability to decolorize molasses melanoidin. Agric. Biol. Chem. 52: 2429-2435.

Pant D, Adholeya A (2007a). Biological approaches for treatment of distillery wastewater: A review. Bioresour.

Technol. 98(12):2321-2334.
Pant D, Adholeya A (2007b). Enhanced production of ligninolytic enzymes and decolorization of molasses distillery wastewater by fungi under solid state fermentation. Biodegradation, 18(5): 647-659
Pant D, Adholeya A (2007c). Identification, ligninolytic enzyme activity and Decolorization potential of two fungi isolated from a distillery effluent contaminated site. Water, Air, Soil Pollut. 183(1-4): 165-176.
Pant D, Adholeya A (2009). Concentration of fungal ligninolytic enzymes by ultrafiltration and their use in distillery effluent decolorization. World J. Microbiol. Biotechnol. 25: 1793-1800.
Pant D, Adholeya A (2010). Development of a novel fungal consortium for the treatment of molasses distillery wastewater. Environmentalist, 30(2): 178-182.
Pearce CI, Lloyd JR, Guthrie JT (2003). The removal of colour from textile wastewater using whole bacterial cells: a review. Dyes Pigm. 58(3): 179-196.
Pena M, Coca M, Gonzalez G, Rioja R, Garcia MT (2003) Chemical oxidation of wastewater from molasses fermentation with ozone. Chemosphere, 51(9): 893-900.
Sapronov AR (1963). Kolichectvennoe opredelenie krasyashchikh veshchestv v produktakh saharnogo proizvodstva (Quantitative determination of colourants in the sugar industry products). Sacharnaja Promyslennost' SSSR (in Russian), 37: 32-35.
Shayegan J, Pazouki M, Afshari A (2005). Continuous decolorization of anaerobically digested distillery wastewater. Proc. Biochem. 40(3-4): 1323-1329.
Strong PJ (2010) Fungal remediation of Amarula distillery wastewater. World J. Microbiol. Biotechnol. 26(1): 133-144.
Tondee T, Sirianuntapiboon S (2008). Decolorization of molasses wastewater by Lactobacillus plantarum No. PV71-1861. Bioresour. Technol. 99(14): 6258-6265.
Wong PK, Yuen PY (1996). Decolorization and biodegradation of methyl red by Klebsiella pneumoniae RS-13. Water Res. 30(7): 1736-1744.
Zayas T, Romero V, Salgado L, Meraz M, Morales U (2007). Applicability of coagulation/flocculation and electrochemical processes to the purification of biologically treated vinasse effluent. Sep. Purif. Technol. 57: 270-276.