Removal of melanoidins by potential manganese peroxidase producing bacteria isolated from molasses effluent

Samir Mahgoub[1,2*], K. Tsioptsias[2], E. Likotrafiti[2] and P. Samaras[2]

[1]Microbiology Department, Faculty of Agriculture, Zagazig University, Zagazig 44511, Egypt

[2]Laboratory of Water and Wastewater Technology, Department of Food Technology, Technological Educational Institute of Thessaloniki, 57400 Sindos, Thessaloniki, Greece

***Corresponding author: email: mahgoubsamir@gmail.com**

Running title: Removal of melanoidins by bacteria isolated from molasses effluent

Abstract

The possibility and efficiency of manganese peroxidase (MP) producing bacteria for the removal of melanoidins from synthetic and real melanoidins solutions were evaluated in this study. Samples collected from bench scale bioreactor treating melanoidins wastewater (90% v/v) were studied for screening and isolation of bacteria capable of decolorizing melanoidins.

Treatment efficiency in terms of color removal, COD reduction, pH change and OD_{590} was investigated under different synthetic melanoidins solutions e.g. sucrose-glutamic acid (SGA), sucrose-aspartic acid (SAA), glucose-aspartic acid (GAA) and glucose-glutamic acid (GGA) Maillard products as well as real melanoidins.

Thirteen potential MP producing bacteria were selected for higher SGA-MP, SAA-MP, GAA-MP and GGA-MP tolerance (7000 mg/l) in modified GPYM Agar (glucose 1.0%, peptone 0.1%, yeast extract 0.1%, K_2HPO_4 0.1 % and $MgSO_4$ 0.05%) and King broth Agar (glycerol 1.0%, peptone 0.1%, yeast extract 0.1%, K_2HPO_4 0.1 % and $MgSO_4$ 0.05%). Three of these isolates and *Lactobacillus kefir* strain showed maximum OD_{590} (1.38- 1.47 and 1.28-1.35) of GAA-MP (5000 and 15000 mg/l) in the medium at pH (7.4 ± 0.1), shaking speed (140 rpm) and temperature (30±2 °C) after 5 days incubation, respectively. However, in real melanoidin solutions (10 % v/v), theses strains showed low OD_{590} (0.43- 0.66) after 5 days incubation, these strains showed maximum decolourisation capacity (8-12 %) of GAA-MP (5000 &15000 mg/l) and real melanoidins (10% v/v) in the medium after 5 days incubation. *Lactob. kefir* and the isolates showed maximum reduction of COD ranged from 52 to 79 % of GAA-MP (5000 & 15000 mg/l) and real melanoidins in the medium after 5 days incubation.

Lactob.kefir and three bacterial isolates showed highest reduction of COD in synthetic and real melanoidins after 5 days of incubation. Yeast extract could be used as bio-stimulator for these isolates during decolourisation of real melanoidins.

Keywords: Melanoidins, Molasses, Decolourisation, microorganisms

Introduction

Molasses distillery wastewater colorants are mainly polyphenols, melanoidin, alkaline degradation products of hexoses, and caramels. A lot of efforts have been made to remove the colorants including biological methods employing different fungi, bacteria &algae, enzymatic treatment, chemical oxidation, coagulation/precipitation, oxidation and membrane filtration [1]. These technologies could also be applied to remove the colorants as a final treatment step after the anaerobic digestion [2]. Melanoidins are found at concentrations of 2.0 % in effluents discharged from molasses processing industry. Untreated distillery effluent or spent wash are well known to cause pollution in the natural streams by increase in organic load, depletion of oxygen content, discoloration and destruction of aquatic life [1]. This effluent retains very dark brown color even after anaerobic treatment due to presence various water soluble, recalcitrant and coloring compounds mainly melanoidins [3]. Melanoidins are generated in sugarcane molasses as complex polymer due to non-enzymatic reaction of sugar and amino acid produced browning reactions called Maillard reaction [4].These compounds are highly resistant to microbial attack, conventional biological processes such as activated sludge treatment are inefficient to decolorize melanoidin-containing wastewaters, such as molasses wastewaters from distilleries and fermentation industries [5]. However, microbial decolorization of anaerobically treated effluent reduced the toxic effect which indicated that there is necessity for microbial degradation at secondary or tertiary stage prior to its disposal for environmental safety [3].Thus, the microbial decolorization can be exploited to develop a cost effective, eco-friendly biotechnology package for the treatment of distillery effluent [6].The decolorization of four types of synthetic melanoidins i.e., glucose–glutamic-acid (GGA), glucose–aspartic-acid (GAA), sucrose–glutamic acid (SGA), and sucrose–aspartic-acid (SAA), were investigated using three different isolates, viz. *Bacillus thuringiensis*, *Bacillus brevis* and *Bacillus* sp. [7].The degree of decolorization of the melanoidins separately by each isolate was in the 1–31% range. The results also indicated that the GAA polymer was the most recalcitrant among the melanoidins tested. The degradation and decolourisation of melanoidin by bacteria are mediated due to prevalence of manganese peroxidase (MnP) as decolourising enzyme [8] and play an important role in degradation of several dyes [9]. *Lactobacillus plantarum* could rapidly decolorize molasses melanoidin to 60.91% and 15.88% color from treated palm oil mill effluent [10]. *Lactob. hilgardii* [11] and *Bacillus* sp [12] had melanoidins pigment removal ability. The decolorization activity of acetogenic bacteria has been reported by Sirianuntapiboon and Prasertsong [13] and showed high decolorization yield in both synthetic melanoidin and molasses wastewater solutions. *Pseudomonas putida* produces hydrogen peroxide which is a strong decolorizing agent. Since the organism cannot use spent wash as a source of carbon, 1% w/v glucose supplement was provided along with 12.5% spent wash. *Aeromonas* sp. utilizes the carbonaceous compounds present in spent wash as the sole carbon source, thereby eventually reducing the effluent COD by 66% in a 24 h period. *P. putida* also resulted in 44% COD removal accompanied by 60% color reduction. In another study on predigested distillery effluent with *A. formicans*, 57% COD reduction and 55% decrease in color was observed after 72 h [14].Therefore, the development of effective and cost-competitive techniques for the decolorization is of importance. Thus the aim of this study was to investigate the synthetic and

real melanoidins adsorption mechanism by *Lactobacillus kefir* and three bacterial isolates from indigenous sources (bench scale bioreactor treating melanoidins wastewater (90% v/v).

2. Materials and methods

2.1. Media and strain used

All the culture media used in this study were of biological grade. Four kinds of media were used i.e. GPYM broth consisting of (w/v): 1% glucose, 0.05% peptone, 0.1% K_2HPO_4 and 0.05% $MgSO_4$, modified GPYM broth amended with synthetic melanoidins solution, King's B' broth media consisting of (w/v): 0.15% K_2HPO_4, O.15% $MgSO_4$, 2.0% Peptone, 1.0% Glycerol and King's B' broth amended with synthetic melanoidins solution. Solid media was prepared by adding 1.5% agar in the above-mentioned composition. *Lactobacillus kefir* strain used in this study was kindly obtained from Dr. Eleni Likotrafiti, Laboratory of Food Microbiology, Department of Food Technology, Technological Educational Institute of Thessaloniki, Greece.

2.2. Preparation of synthetic melanoidins

The synthetic melanoidins solution used in this study was prepared by refluxing the equimolar (1M) solution of sucrose (Panreac Quimica SAU, Barcelona, Spain), aspartic acid (Panreac Quimica SAU, Barcelona, Spain) and 0.5M sodium carbonate at 100 ◦C for 7 h [7] whereas; natural melanoidins solution was prepared from distillery effluent. Four types of melanoidins i.e., sucrose-aspartic-acid (SAA), sucrose-glutamic acid (SGA), glucose-aspartic-acid (GAA) and glucose-glutamic-acid (GGA) were synthesized SAA was synthesized using sucrose, aspartic acid and sodium carbonate, SGA from sucrose, glutamic acid and sodium carbonate, GAA from glucose, aspartic acid and sodium carbonate and GGA was prepared utilizing glucose, glutamic acid and sodium carbonate under above conditions and then after adjustment of the reaction mixture to pH 7.4 with 1N NaOH. These solutions contained mg/l COD from 267000 to 418000 mg/l.

2.3. Isolation of potential manganese peroxidase producing bacteria

Samples collected from bench scale bioreactor treating melanoidins wastewater (90% v/v) were studied for screening and isolation of bacteria capable of decolorizing melanoidins. About 5 g of melanoidins wastewater was transferred to a conical flask having capacity 250 ml containing sterile synthetic melanoidins solution (3000 mg/l) in modified GPYM medium (95 ml) at pH 7.0 and 0.1% phenol red (w/v) [15]. The flasks were incubated at 30±2 ◦C in a rotary shaking incubator at 140 rpm for 6 days. When decolourisation was observed in samples, an aliquot (100µl) was spread onto GPYM or King's B agar plates amended with synthetic melanoidins solution (3000 mg/l) and incubated at 30±2 ◦C for 48 h. The morphologically different colonies growing on plates were further purified onto GPYM agar plates amended with synthetic melanoidins (3000 mg/l). The purity of each bacterial culture was checked under the microscope. For short term storage, these strains were maintained on GPYM agar slants at 4 °C and for long term storage GPYM broth glycerol stocks were prepared and kept at -80 °C.

2.4. Screening of potential isolates for manganese peroxidase activity

The bacterial isolates and *Lactob kefir* strain were screened on the basis of growth and peroxidase activity on modified GPYM agar plates amended with different concentrations of synthetic melanoidin solution GAA (3000, 4000, 5000, 6000 and 7000 mg/l) and 0.1% phenol red (w/v). The bacterial isolates and strain showed optimum growth and exhibited peroxidase activity by changing the deep red colour of dye to light yellow. Three bacterial strains B1, B2 and B3 showed exhibiting peroxidase activity (e.g. capability to degrade melanoidin compounds). These isolates and *Lactob. kefir* were used in subsequent experiments at 5000 and 15000 mg/l synthetic GAA melanoidin in GYPM at 30±2 ◦C as well as at 10% v/v real melanoidins in yeast extracts (0.1% w/v).

2.5. Inoculum preparation and decolourisation studies

The inoculum was prepared in two steps. In the first stage, a pure culture was transferred to a 50 ml modified GPYM broth consisting of (w/v): 1.0% glucose, 0.05% peptone, 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, incubated for 24 h in shaking flasks (140 rpm) under aerobic condition at 30 ±2 °C. Subsequently, it was transferred into shaking flasks containing synthetic melanoidin (3000 mg/l) mended GPYM broth. In the first experiment, the decolourisation experiments were carried out by the addition of bacterial inoculum (OD_{590} 0.05- 0.08) in 250 ml flasks containing 50 ml modified GPYM broth containing GAA with concentration of 5000 or 15000 mg/l under shaking flask conditions (140 rpm) at pH 7.4 and 30 ±2 °C for 5 days. A separate set of uninoculated flasks was maintained in parallel as control. In the second experiment *Lactob. kefir* and three bacterial isolates were assessed for their decolorization potential in natural melanoidins pigment (10% (v/ v)) amended with yeast extract (0.1% w/v) under shaking flask conditions (140 rpm) at pH 7.4 and 30 ±2 °C for 5 days. The addition of bacterial inoculum OD_{590} was 0.05- 0.06 in 250 ml flasks containing 50 ml real melanoidins (10% v/v) and 90% sterilized water containing yeast extract (0.1% w/v) under shaking flask conditions (140 rpm) at pH 7.4 and 30 ±2 °C. A separate set of uninoculated flasks was maintained in parallel as control. Samples were withdrawn from culture media at regular 24 h intervals for decolourisation measurements. The supernatant was taken after centrifugation (10000g for 10 min) for determination of color and chemical oxygen demand (COD) [16]. Melanoidins removal or adsorption activity was determined as a decrease of optical density in the absorbance at 400 to 800 nm against the control. The PH was determined in supernatant. The cell pellet obtained on centrifugation was resuspended in 5ml distilled water and its absorbance was measured at OD_{590} nm and reported as growth of the strains. Experiments were repeated three times to reduce experimental error.

3. Results and discussion

3.1. Bacterial isolation and screening for manganese peroxidase activity

Thirteen morphologically distinct bacterial strains (B1 to B13) isolates were identified to be 6 Gram negative and 10 Gram-positive. These bacterial cultures and *Lactob. kefir* strain were screened on the basis of growth and peroxidase activity shown in GPYM agar plates amended with different concentrations of synthetic melanoidins (3000, 4000, 5000, 6000 and 7000 mg/l) with 0.1 %(w/v) phenol red (Table 1). Results revealed that all the strains showed

growth at all concentrations (3000, 4000, 5000, 6000 and 7000 mg/l v/v). The isolates and strain showed fast growth and peroxidase activity up to 5000 mg/l GAA in terms of change in color from deep orange to light yellow within 48 h incubation periods at 30 °C. However, out of thirteen isolated bacterial strains, B1, B2 and B3 only showed fast growth and peroxidase activity at all concentrations of GAA. On the other hand, bacterial strains B4 to B13 showed only moderate growth at 6000 and 7000 mg/l of synthetic melanoidins, but no peroxidase activity even after 120 h. The same observation has been also reported by Yadav et al., [17]. Phenol red-amended GPYM agar plates used as indicator of peroxidase activity shifted its color from deep orange to light yellow and this decolorization of phenol red has been used as an indicator of peroxidase activity [18]. This change in color of phenol red-amended GPYM agar medium resulted in the oxidation of glucose, leading to the generation of H_2O_2 and media acidification (i.e. lowering of pH) because during the melanoidin degradation, peroxidase activity required H_2O_2, which was produced during the glucose oxidation by sugar oxidase enzymes [19].

3.2. Decolourisation studies of SAA, GAA and real melanoidins

The change of pH values during the decolorization of SAA, GAA and natural melanoidin at 32±2 °C is shown in Fig. 1. The pH 7.2± 0.2 noted favorable for SAA, GAA and real melanoidins decolourisation after 24 h incubation at 32±2 °C. While further, after 48 to 120 h the pH values were decreased up to 5 in case of GAA and were increased and reached the maximum 8.2 in case of SAA and real melanoidins. The acidic and alkaline pH in melanoidins solution showed reduction in melanoidins decolourisation. This result concluded that this could be as a result of maximum melanoidins solubility at neutral pH. These results explained that the increase or decrease in pH to alkaline or acidic adversely affected the growth and decolourisation ability of the strains. Also,the strains might be loss of MnP enzyme activity at an alkaline or acidic pH values. Thus, it can be concluded that the environmental factors like pH, temperature, aeration and nutrients play a vital role in microbial degradation process of industrial wastes because the activity of enzymes is greatly influenced by them various environmental factors.

The optical density (OD) for bacterial growth at 590 nm was observed during the degradation of SAA (5 ml/l) and real melanoidins (%10 v/v) and is presented in Fig.2. The results revealed that a marked increase in OD_{590} for all bacterial growth and reached the maximum growth after 2 days of incubation. Three of these isolates and *Lactobacillus kefir* strain showed maximum OD_{590} (1.38- 1.47 and 1.28- 1.35) of GAA-MP (5000 and 15000 mg/l) in the medium at pH (7.4 ± 0.1), shaking speed (140 rpm) and temperature (30±2 °C) after 5 days incubation, respectively. However, in real melanoidin solutions (10 % v/v), theses strains showed low OD_{590} (0.43- 0.66) after 5 days incubation. This increased in the bacterial growth was in parallel with the degradation of SAA and real melanoidins. Moreover, the bacterial culture was found more effective for the degradation of melanoidins after 1 day of incubation, as it decolorized 12 and 8 % SAA (15 ml/l) and natural melanoidins (Fig. 3) respectively. The same trend was observed in all bacterial cultures. It was noticed that the decolorization of melanoidins decolorization by *Lactob. kifer* strains and three bacterial isolates might be due to the cell growth associated. *Lactob. plantarum* SF5.6 grew rapidly within one day while the other strains took 2–3 days [10]. Also, the decolorization of four types of synthetic melanoidins i.e., glucose–glutamic-acid (GGA), glucose–aspartic-acid

(GAA), sucrose–glutamic acid (SGA), and sucrose–aspartic-acid (SAA), were investigated using three different isolates, *B. thuringiensis*, *B. brevis* and *Bacillus* sp. [7].The degree of decolorization of the melanoidins separately by each isolate was in the 1–31% range. After 2 days of incubation with the bacterial culture, there was no decreased in decolorazation. It was noticed that with the increase in time duration, decolorization activity of the three isolate B1, B2 and B3 was decreased up to 5th day (Fig. 3). This was explained by the fact that the pH affected the metabolic value (microbial acceptability) by generating intermediate hydrolysis products from the compounds present in the SAA and real melanoidens. The biodegradability was further enhanced by combined SAA with GPYM broth or real melanoidens with yeast extracts resulting in 34 to 66 % COD reduction after 2 days as compared to the control (Fig. 4). *Lactob. kefir* and the isolates showed maximum reduction of COD ranged from 52 to 79 % of SAA (5000 & 15000 mg/l) and real melanoidins in the medium after 5 days incubation. *Aeromonas* sp. utilizes the carbonaceous compounds present in spent wash as the sole carbon source, thereby eventually reducing the effluent COD by 66% in a 24 h period. *P. putida* also resulted in 44% COD removal accompanied by 60% color reduction. In another study on predigested distillery effluent with *A. formicans*, 57% COD reduction and 55% decrease in color was observed after 72 h [14]. The enhancement in biodegradability was attributed to the source of bacterial isolation which isolated from molasses wastewater treatment. The decolorization of melanoidin by the bacterial isolates and *Lactob. kifer* was shown to be closely related to the composition of medium and the concentration of melanoidins.

Conclusion

Lactob.kefir and three bacterial isolates showed highest reduction of COD in synthetic and real melanoidins after 5 days of incubation. Yeast extract could be used as bio-stimulator for these isolates during decolourisation of real melanoidins. These isolates could be useful for decolourisation of industrial wastewater containing high concentration of melanoidins.

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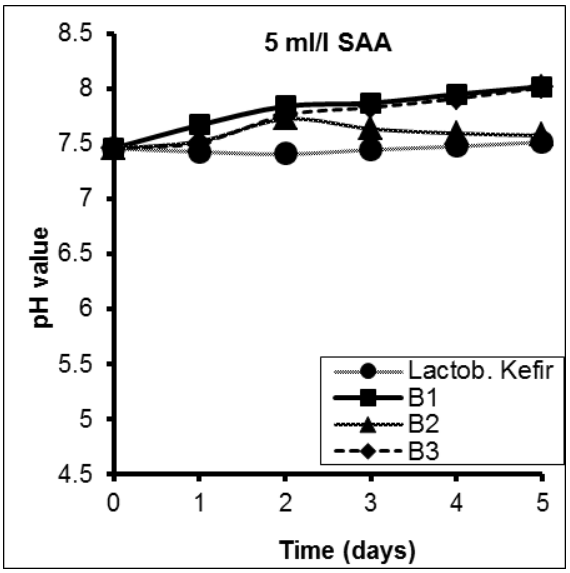
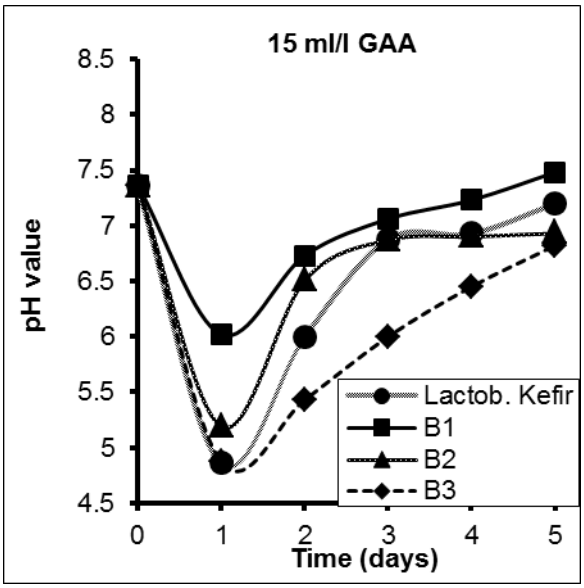
Table 1: Selection of potential bacterial isolates on the basis of growth pattern and enzyme activity on SA-MP, SG-MP, GA-MP and GG-MP amended GPYM media at different concentrations.

Melanoidines (mg/l)	Bacterial isolates													
	Lactobacillus kefir	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**
SAA-MP														
3000	++***	+++** *	+**	+**	++*	++***	++***	++***	+++***	++*	+++***	+++***	+++***	+++***
4000	+++** *	+++** *	+**	+**	+	+++** *	+++** *	+++** *	+++***	+	+++***	+++***	+++***	+++***
5000	+++**	++***	+**	+**	+	+++**	+++**	+++*	+++*	+	+++**	+++**	+++*	++**
6000	++	++**	+**	+**	+	++	++	++	++	+	++	++	++	++
7000	+	+**	+**	+**	+	+	+	+	+	+	+	+	+	+
SGA-MP														
3000	+++** *	+++** *	+**	+**	++*	+++** *	+++** *	+++** *	+++***	++*	+++***	+++***	+++***	+++***
4000	+++** *	+++** *	+**	+**	+	+++** *	+++** *	+++** *	+++***	+	+++***	+++***	+++***	+++***
5000	+++**	++***	+**	+**	+	+++**	+++**	+++*	+++*	+	+++**	+++**	+++*	++**
6000	++	++**	+**	+**	+	++	++	++	++	+	++	++	++	++
7000	+	+**	+**	+**	+	+	+	+	+	+	+	+	+	+
GAA-MP														
3000	+++** *	+++** *	+**	+**	++*	+++** *	+++** *	+++** *	+++***	++*	+++***	+++***	+++***	+++***
4000	+++**	+++**	+**	+**	+	+++**	+++**	+++**	+++***	+	+++***	+++***	+++***	+++***

	*	*				*	*	*						
5000	++ +**	++ ***	+**	+**	+	++ +**	++ +**	++ +*	+++ *	+	+++ **	+++ **	+++ *	++* **
6000	++	++ **	+**	+**	+	++	++	++	++	+	++	++	++	+
7000	+	+**	+**	+**	+	+	+	+	+	+	+	+	+	+
GGA-MP														
3000	++ +** *	++ +** *	+**	+**	++ *	++ +** *	++ +** *	++ +** *	+++ ***	++*	+++ ***	+++ ***	+++ ***	+++ ***
4000	++ +** *	++ ***	+**	+**	+	++ +** *	++ +** *	++ +** *	+++ ***	+	+++ ***	+++ ***	+++ ***	+++ ***
5000	++ +**	++ **	+**	+**	+	++ +**	++ +**	++ +*	+++ *	+	+++ **	+++ **	+++ *	++* **
6000	++	+**	+**	+**	+	++	++	++	++	+	++	++	++	++
7000	+	+**	+*	+*	+	+	+	+	+	+	+	+	+	+

SA-MP=Sucrose-aspartic acid, SG-MP= Sucrose-glutamic acid, GA-MP= Glucose-aspartic acid and GG-MP= Glucose-glutamic acid Maillard product; += growth; *= enzyme activity





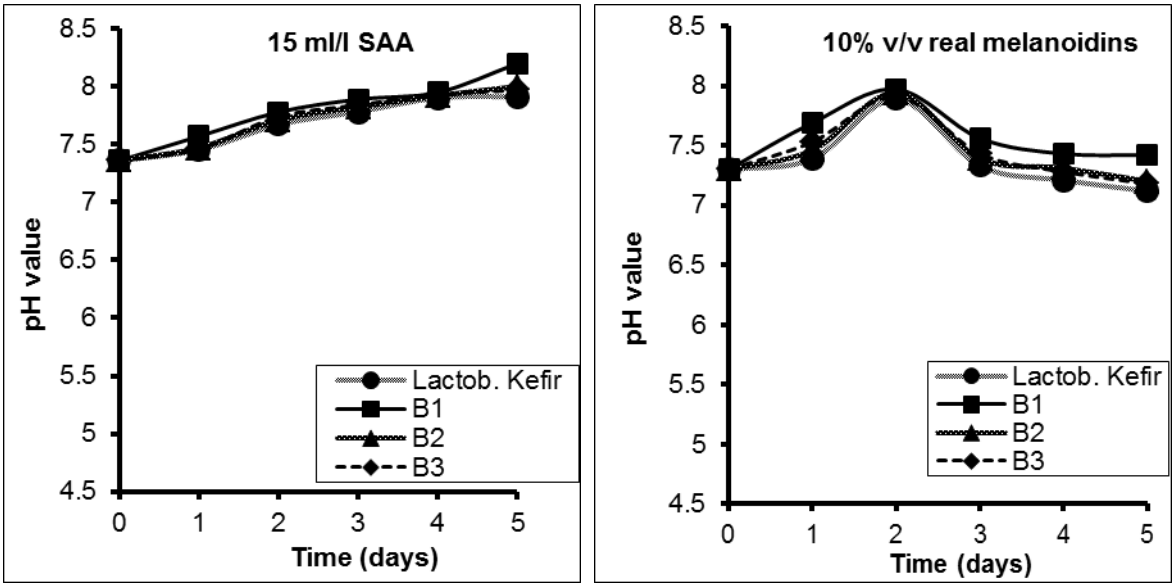
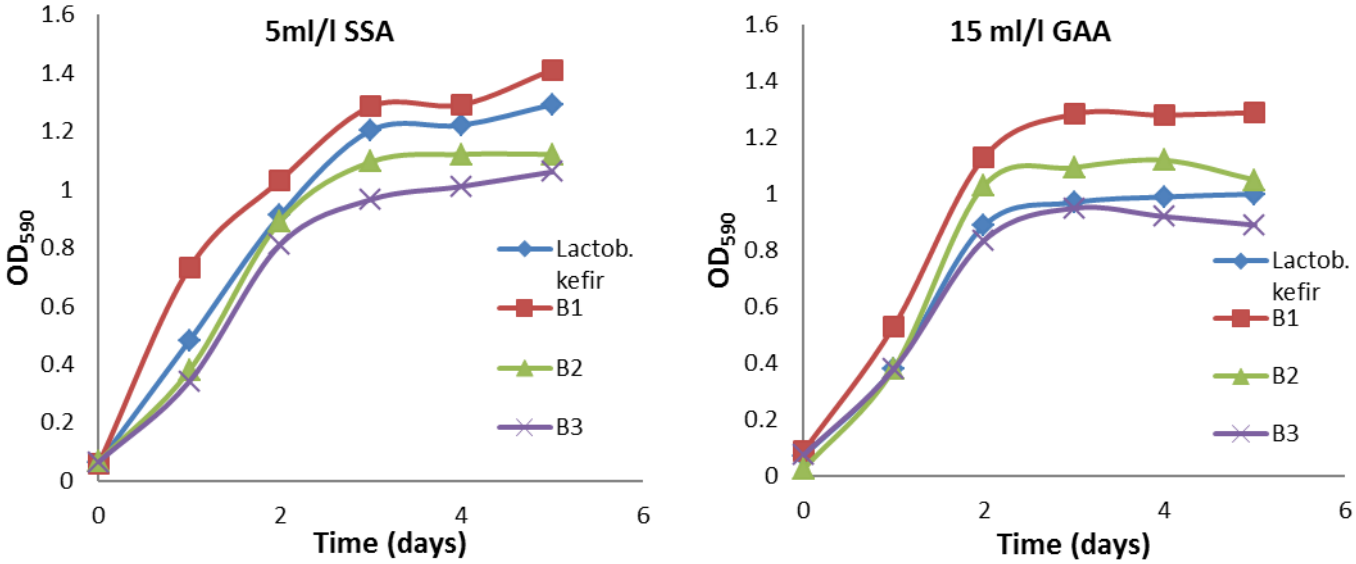


Fig. 1 Change in pH values of synthetic (SSA or GAA) and real melanoidins by different bacterial strains during their growth for decolorization



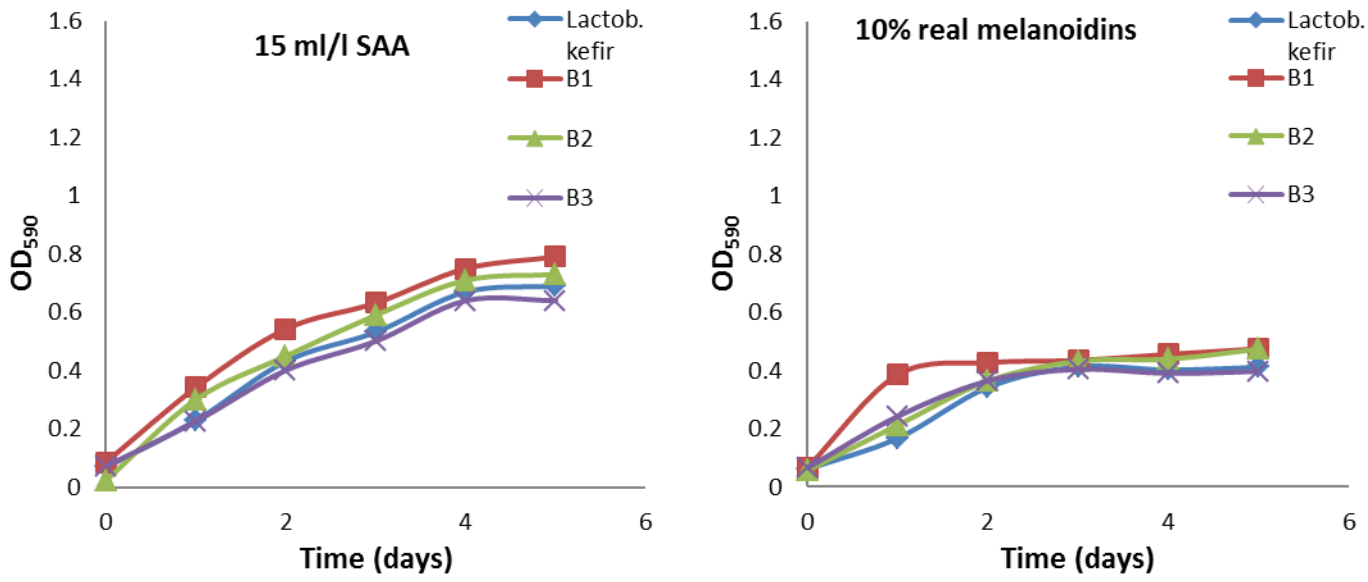
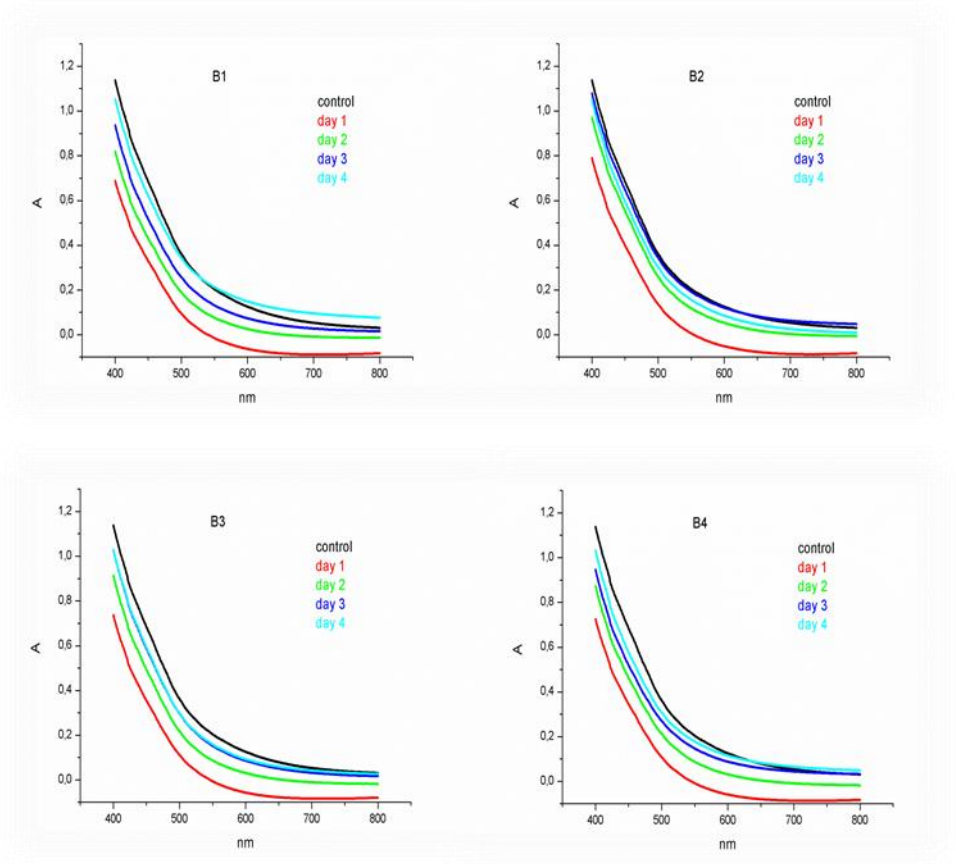


Fig. 2 Change in OD_{590} values of different bacterial strains during decolorization of synthetic (SSA or GAA) and real melanoidins

Fig. 3. The decolorization of synthetic melanoidin (SSA) 5m/l by different bacterial strains, B1: Lactobacillus kefir, B2, B3 and B4 bacterial isolates from molasses wastewater treatment bench scale



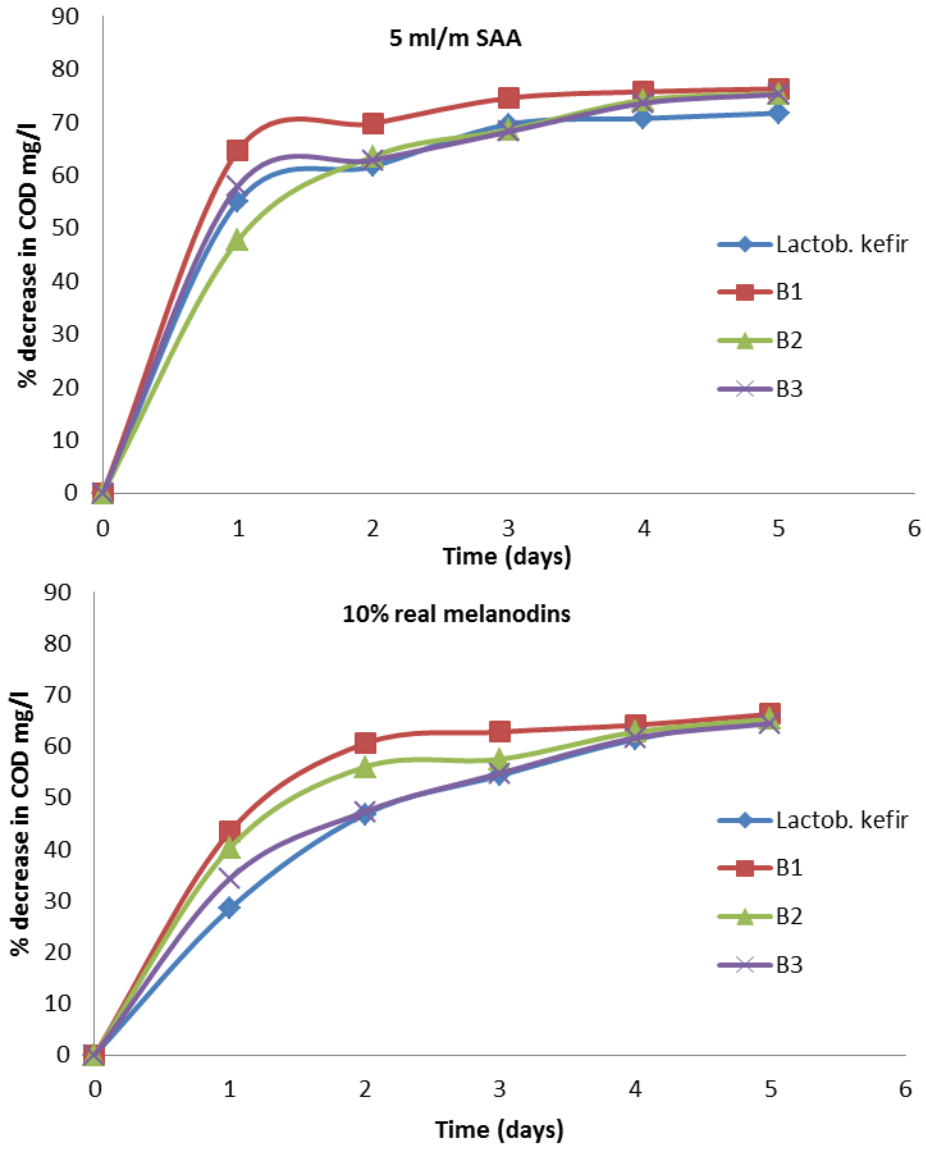


Fig. 4 Change of COD mg/l in synthetic and real melanoidins by different bacterial strains