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Decolorization of Distillery Effluent by Thermotolerant *Bacillus subtilis*

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Abstract: Problem statement: Ethanol production from sugarcane molasses generate large volume of effluent containing high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) along with melanoidin, a color compound generally produced by "Millard reaction". Melanodin is a recalcitrant compound degraded by specific microorganisms having ability to produce mono and di-oxygenases peroxidases, phenoxidases and laccases, are mainly responsible for degradation of complex aromatic hydrocarbons like color compound. These compounds causes several toxic effects on living system, therefore may be treated before disposal. **Approach:** The purpose of this study was to isolate a potential thermotolerant melanoidin decolorizing bacterium from natural resources for treatment of distillery effluent at industrial level. **Results:** Total 10 isolates were screened on solid medium containing molasses pigments. Three potential melanoidin decolorizing thermotolerant bacterial isolates identified as *Bacillus subtilis*, *Bacillus cereus* and *Pseudomonas* sp. were further optimized for decolorization at different physico-chemical and nutritional level. Out of these three, *Bacillus subtilis* showed maximum decolorization (85%) at 45°C using (w/v) 0.1%, glucose; 0.1%, peptone; 0.05%, $MgSO_4$; 0.01%, KH_2PO_4; pH-6.0 within 24h of incubation under static condition. **Conclusion/Recommendations:** The strain of *Bacillus subtilis* can tolerate higher temperature and required very less carbon (0.1%, w/v) and nitrogen sources (0.1%, w/v) in submerged fermentation. It can be utilized for melanoidin decolorization of distillery effluent at industrial scale.

Key words: Spentwash, melanoidin, millard reaction, *Bacillus subtilis*

INTRODUCTION

Sugar industry produced, several by-products such as molasses, bagasse and fiber cake, among which molasses is the most important. Molasses contains about 48-50% sucrose and has a high viable value due to its use as a carbon source in various fermentation processes and also as livestock feed and biofertilizer (Dahiya *et al*., 2001; Pazouki *et al*., 2008). Molasses use as a raw material by distilleries for ethanol production which produces dark brown color spentwash with a high Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), low pH and toxic substances such as phenols (Dahiya *et al*., 2001; Fitzgibbon, 1995). The dark color remains as a problem which requires a pretreatment before its safe dumping into the environment. The main problem in treating Distilleries Spentwash (DS) is its color, which contains nearly 2% (w/w) of a dark brown recalcitrant pigment, melanoidin (Pazouki *et al*., 2008). Melanoidin is known as a natural browning polymer, produced by the "Maillard reaction" between amino and carbonyl groups of organic matters and is closely related to humic substances in the natural environment (Wedzicha and Kaputo, 1992; Fujita *et al*., 2000).

The disposal of distillery spentwash into the environment is toxic, lead to a reduce in sunlight penetration in rivers, lakes or lagoons, which in turn, decreases both photosynthetic activity and dissolved oxygen concentrations causing harm to aquatic life (Pazouki *et al*., 2008). Disposal on land is evenly hazardous, causing reduce in soil alkalinity and also in soil manganese availability inhibition of seed germination and vegetation growth (Agrawal and Pandey, 1994). Several physico-chemical and biological methods have been examined for decolorization of distillery spentwash. Melanoidins can be removed by physico-chemical treatments but these methods require high reagent dosages and generate large amount of sludge (Pena *et al*., 2003; Mohana *et*

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al., 2007). Biological methods present an incredible alternate for decolorizaiton/degradation and bioremediation of spentwash due to their low cost, environmental friendly and publicly acceptable treatment and cost competitive alternative to chemical decomposition processes (Moosvi *et al*., 2005; Mohana *et al*., 2007).

A number of biological processes such as bioadsorption and biodegradation have been reported having prospective application in color removal from spentwash (Ohmomo *et al*., 1987; Kumar *et al*., 1997; Kumar and Chandra, 2006; Plavsic *et al*., 2006; Pant and Adholeya, 2007; Nwuche and Ugoji, 2008; 2010). A wide variety of aerobic microorganisms capable of decolorizing spentwash include bacteria, fungi, cyanobacteria and yeasts. Some bacterial strains isolated from sewage and acclimatized on increasing concentrations of distillery waste, which were able to reduce Chemical Oxygen Demand (COD) by 80% in 4-5 days without any aeration and the major products left after the degradation process were biomass, carbon dioxide and volatile acids (Kumar and Viswanathan, 1991). Raghukumar and Rivonkar (2001) isolated a marine fungus, *Flavodon flavus*, which was more effective in decolorizing raw molasses spentwash than was the molasses wastewater collected either after anaerobic treatment or after aerobic treatment. Tondee and Sirianutapiboon (2006) isolated *Issatchenkia orientalis* yeast from fruit sample showed 60% melanoidin decolorization at 30°C in 7 days under aerobic condition.

In the present investigation, an attempt was made to isolate such strains from natural ecosystem which has ability to grow at higher temperature without requirement of simple sugar and higher percentage of melanoidin decolorization even reported.

MATERIALS AND METHODS

Distillery Spent Wash (DSW): The molasses spent wash was collected aseptically from Masuadh sugarcane distillery India. The spentwash was centrifuged at 10,000 rpm for 15 min before use to remove the suspended solids and stored at 4°C (Pazouki *et al*., 2008). The stored distillery spentwash was filtered through (What man No: 1) filter paper and was diluted with distilled water. The analysis of different physico-chemical parameters like color, odour, pH, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), total sugars, Total Dissolved Solids (TDS), sulphates, phosphorous and calcium were analysed for employing standard methods for examination of water and wastewater (Eaton and Franson, 2005), result is shown in Table 1.

Table 1:Physico-chemical properties of distillery effluent (spentwash) All parameter are in mg/L

Parameters	Value of distillery effluent
Color	Dark brown
Odour	Like molasses
Temperature °C	82.0
pH	4.2
Total dissolved solid	81733.0
Total suspended solid	5933.0
Dissolved oxygen	0.0
Biological oxygen demand	46666.0
Chemical oxygen demand	104130.0
Total nitrogen	1635.0
Phosphorus	163.0
Potassium	8766.0
Sodium	211.0
Calcium	1816.0
Sulphate	1738.0

Isolation, screening and identification of melanoidin decolorizing bacteria: Melanoidin decolorizing bacteria isolated from soil sample collected from Masaudh sugarcane distillery Faizabad, India, was grown on GPYE medium for 24-48 h incubation. Culture medium consisted of 0.01%, KH_2PO_4; 0.05%, $MgSO_4.12H_2O$; 0.5%, glucose and 0.1%, yeast extract with 3.5 OD effluent and the initial pH was adjusted to 6.0. In order to isolate molasses-decolorizing bacteria, 1g of soil was serialy dilution upto 10^{-5}-10^{-6} and placed in Petri-plates along with the basal agar medium. The plates were subsequently incubated for 24-48 h at 35±2°C and 45±2°C for thermotolerant bacteria. After 24-48 h of incubation decolorization effect was seen visually. The isolates showing higher decolorization of the melanoidin were selected for further studies, maintained on the same medium at 4°C in slants and sub-cultured after 15 days. The cultures were identified at genus and species level by IMTECH Chandigarh, India.

Inoculum preparation: Cell suspension was prepared by inoculating 1 mL of 24 h grown culture in 50 mL basal broth and then incubated at 35°C for 24 h to achieve active exponential phase of culture consisting 5×10^6cfu/mL transfered into the flask and incubated in static condition. Quantitative decolorization value was determined on the basis of OD at 475 nm against the blank by UV-visible Spectrophotometer (Shimadzu UV-VIS modal 1601, Japan).

Decolourization assay of the spent wash: The melanoidin decolorizing bacterial isolates were inoculated in the basal broth medium and after incubation; broth was centrifuged at 10,000 rpm for 10 min. The supernatant of the centrifuged sample will read at absorbance maximum (A_{max}) of the melanoidin i.e., 475 nm using spectrophotometer (Ohmomo *et al*., 1988). The decolorization yield will be expressed as the decrease in the absorbance at 475 nm against initial

absorbance at the same wavelength. Uninoculated medium will serve as control. The entire assay were performed in triplicate and compared with control. The decolourization efficiency of the different isolates will be expressed as per following equation:

Decolourization (%) = I - F / I

Where:
I = Initial absorbance (Control) and
F = Absorbance of decolourized medium broth

Optimization of culture conditions for decolourization: Selection of physical parameters for melanoidin decolorization: The basal medium for melanoidin decolorization with different temperature viz. 35, 40, 45, 50 and 55°C and incubation period viz. 8, 16, 24, 32 and 40 h were used for the melanoidin decolorization. The initial pH (6.0) was varied in the medium by adding either 1N HCl or 1N NaOH as required. The basal medium was then inoculated with 0.5% (v/v) inoculum of bacterial isolates having $5x10^6$cfu/mL population respectively and incubated at different pH viz. 5.0, 5.5, 6.0, 6.5 and 7.0 for optimization of melanoidin decolorization.

Selection of nutritional parameters for melanoidin decolorization: Various carbon sources viz. glucose, fructose, sucrose and lactose at 0.5% (w/v) were individually added in the basal medium and inoculated with 0.5% (v/v) of bacterial cultures separately with their respective optimized pH, temperature then incubated for 24h for decolorization. The best source of sugar will further optimized in different concentration viz. 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6% (w/v) for melanoidin decolorization.

In another experiment, different organic and inorganic nitrogen sources viz. beef extract, yeast extract, peptone, ammonium sulphate and ammonium nitrate were individually added into the basal medium at 0.5% (w/v). Active culture of individual bacteria was inoculated with 0.5% (v/v) inoculum having 5×10^6cfu/mL. The best source of nitrogen will further optimized in different concentration viz. 0.1, 0.2, 0.3, 0.4 and 0.5% (w/v) for melanoidin decolorization.

Statistical analysis: All the experiments were carried out in triplicates and the results are presented as the mean of three independent observations. Standard deviation for each experimental result was calculated using Microsoft Excel.

RESULTS

Isolation, screening and identification of melanoidin decolorizing bacterial isolates: A total of 10 bacterial isolates showing decolorization ability were isolated on the basal agar medium from the soil of distillery near by the Masudha distillery Faizabad, on qualitative basis. The isolates showing higher clear zone around the colony on molasses agar were selected, at pH 6.0 for 24-48h at 45°C. The clear zone diameter of more than 1 cm around the colony was considered as effective isolates for decolorization (data not shown).

Secondary screening was made on quantitative basis using melanoidin broth medium containing molasses wastewater with distilled water to 3.5 OD consisted of 0.01%, KH_2PO_4; 0.05%, $MgSO_4.12H_2O$; 0.5%, glucose and 0.1%, yeast extract with initial pH 6.0. Each isolates were inoculated in 50 mL of medium in 250 mL Erlenmeyer flask and kept for incubation at 35 and 50°C for 48 h for selection of thermotolerant melanoidin decolorizing bacteria individually. Among bacterial isolates, higher decolorization was shown by three selected bacterial isolates. However, these bacterial isolates were studied for higher decolorization at different physico-chemical and nutritional parameters.

The bacterial cultures were identified by IMTECH, Chandigarh shown in Table 2 and identified as *Bacillus subtilis* (MTCC, 2819), *Bacillus cereus* (MTCC, 3691) and *Pseudomonas aeruginosa* (MTCC, 10181).

Optimization of different physico-chemical and nutritional parameters for melanoidin decolorization: Effect of different temperature on melanoidin decolorization: Effect of different temperature viz. 35-55°C was evaluated for melanoidin decolorization by three different bacterial strains at different physico-chemical and nutritional levels. *Bacillus subtilis* showed best decolorization (72%) at 40°C and even upto 50°C, showing best thermotolerance ability as compared to *Bacillus cereus* (65%) and *Pseudomonas sp*. (68%) at 45 and 35°C respectively. Further, increase in temperature, could not affect decolorization efficiency by the strains (Fig. 1).

Effect of different incubation on melanoidin decolorization: Just after optimization of temperature for melanoidin decolorization in the liquid medium, incubation period was simultaneously optimized for decolorization. The results clearly indicated that *Bacillus subtilis* showed 72% decolorization in 24 h of incubation. Further increase in the incubation period did not increase the decolorization (Fig. 2). *Bacillus cereus* showed the 67% decolorization in 32 h of incubation while *Pseudomonas* sp. showed the highest decolorization (68%) in 40 h. Therefore, *Bacillus subtilis* showed higher decolorization in short time period in comparison to other.

Table 2: Identification of distillery effluent (spentwash) decolorizing bacteria TSI- Triple sugar iron, A- Acid, AG- Acid gas, H_2S - Hydrogen sulphite, (-) Negative, (+) Positive

Test	B 1	B 2	B 3
Gram's nature	+	+	_
Shape	Rod	Rod	Rod
Motility	Motile	Motile	Motile
Glucose fermentation	A	AG	_
Sucrose fermentation	A	A	_
lactose fermentation	A	A	_
Maltose fermentation	A	A	A
Mannitol fermentation	A	_	_
TSI	A/A, H_2S	A/A, H_2S	A, H_2S
Indol production	_	_	_
Methyl red	_	_	_
Voges-Prausker	+	_	_
Citrate utilization	+	_	+
Catalase	+	+	_
Oxidase	_	_	+
Isolate Identification	*Bacillus subtilis*	*Bacillus cereus*	*Pseudomonas aeruginosa*

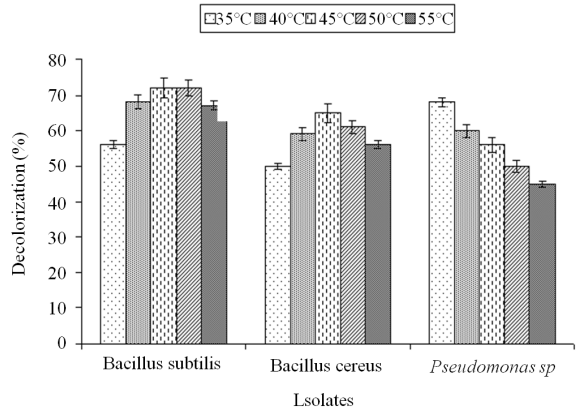

Fig. 1: Effect of different temperature on melanoidin decolorization. The inoculated flasks were incubated at different temperature (°C) for 24-48 h at static condition in medium. Error bars presented are mean values of ± standard deviation of triplicates

Effect of pH on color removal: Different pH viz. 5.0-7.0 in the basal medium was evaluated for melanoidin decolorization by the bacteria at their optimal temperature and incubation periods. *Bacillus subtilis* showed higher 76% decolorization at pH 6.0. *Bacillus cereus* showed the 69% decolorization at 6.5 while *Pseudomonas* sp. showed the highest decolorization (68%) at pH 7.0 (Fig. 3) Further, increase and decrease in the medium pH reduced the decolorization.

Effect of different carbon sources on melanoidin decolorization: Various carbon sources viz. sucrose, glucose, fructose and lactose at a concentration of 0.5% were individually tested in the basal medium at their optimal temperature, incubation period and pH to observe the effect on melanoidin decolorization by the bacteria.

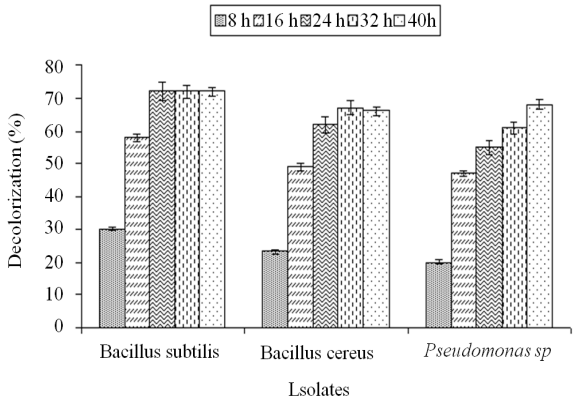

Fig. 2: Effect of different incubation periods on melanoidin decolorization. The inoculated flasks were incubated at different incubation period at 40°C under static condition in medium. Error bars presented are mean values of ± standard deviation of triplicates of three independent experiments

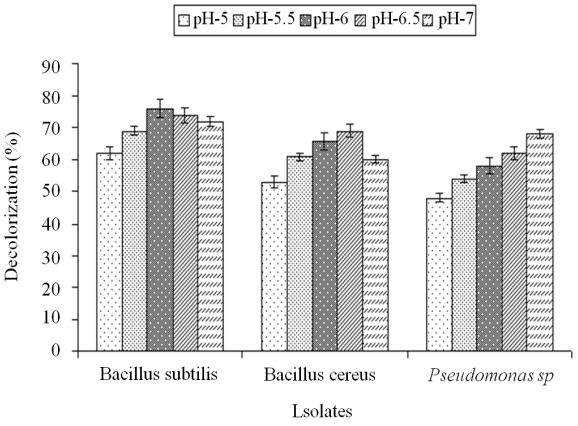

Fig. 3: Effect of different pH on melanoidin decolorization. The inoculated flasks were incubated at different pH at 40°C for 24 h under static condition in medium. Error bars presented are mean values of ± standard deviation of triplicates of three independent experiments.

Out of these carbon sources, glucose was found best for melanoidin decolorization by the bacteria followed by fructose. Higher decolorization (76%) was reported by *Bacillus subtilis*, fructose, sucrose favoured the decolorization. While *Bacillus* cereus and *Pseudomonas sp*. showed 70 and 66% decolorization with glucose also. *Bacillus subtilis* was least affected and showed less affinity regarding decolorization (Fig. 4).

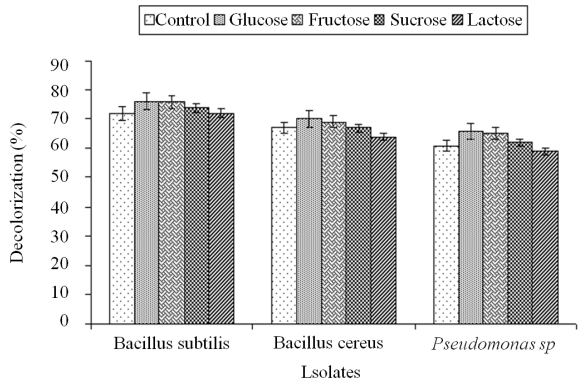

Fig. 4: Effect of different carbon sources on melanoidin decolorization. The control flask does not contain any carbon sources. Test flasks contained different carbon sources in the medium at a level of 0.5 % (w/v). Inoculated flasks were incubated at 40°C for 24 h. Error bars presented are mean values of ± standard deviation of triplicates of three independent experiments

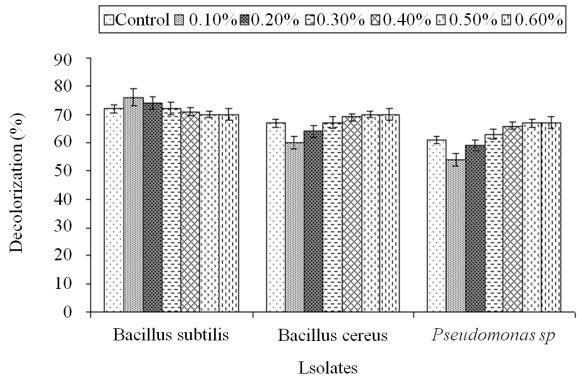

Fig. 5: Effect of different glucose concentration on melanoidin decolorization. The control flask does not contain glucose. Test flasks contained different concentration of glucose in the medium at a level of 0.6 % (w/v). Inoculated flasks were incubated at 40°C for 24 h. Error bars presented are mean values of ± standard deviation of triplicates of three independent experiments

Effect of different concentration of glucose on melanoidin decolorization: In another set of the experiment, different concentrations of glucose (0.1-0.6%) in the medium were tested for melanoidin decolorization at the same growth conditions at which carbon sources were evaluated. *Bacillus subtilis* showed 76% decolorization at 0.1% glucose concentration, while *Bacillus cereus* and *Pseudomonas sp*. were showed 70% and 67% decolorization at 0.5% concentration of glucose.

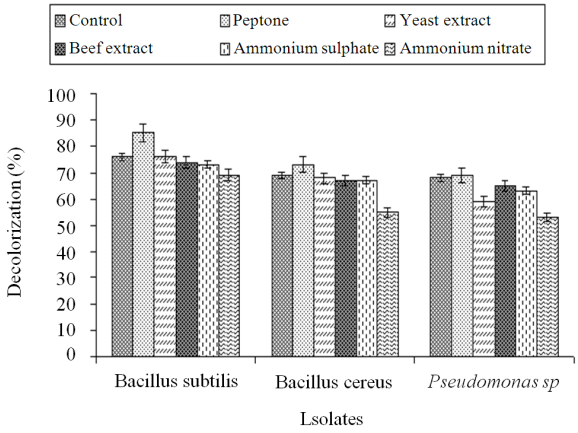

Fig. 6: Effect of different nitrogen sources on melanoidin decolorization. The control flask does not contain any nitrogen sources. Test flasks contained different nitrogen sources in the medium at a level of 0.5 % (w/v). Inoculated flasks were incubated at 40°C for 24 h. Error bars presented are mean values of ± standard deviation of triplicates of three independent experiments

Bacillus subtilis was found to be the most effective decolorizer when compared with *Bacillus cereus* and *Pseudomonas* sp. Above and below of this concentration decolorization reduced and biomass was slightly increased (Fig. 5).

Effect of different nitrogen sources on melanoidin decolorization: Inorganic and organic nitrogen viz. beef extract, yeast extract, peptone, ammonium sulphate, ammonium nitrate, at the rate of 0.5% were used in the basal medium for melanoidin decolorization by the bacteria (Fig. 6). The melanoidin decolorization by the bacteria was almost similar in peptone amended medium, while other nitrogen sources did not increase in decolorization percentage. *Bacillus subtilis* was showed 85% decolorization with peptone while *Bacillus cereus* and *Pseudomonas sp*. were showed only 73 and 69% decolorization respectively.

Effect of different concentration of peptone on melanoidin decolorization: Different concentrations of peptone (0.1, 0.2, 0.3, 0.4 and 0.5 %) in the medium were also tested for melanoidin decolorization at the same growth condition at which nitrogen sources were evaluated. *Bacillus subtilis* showed better decolorization (85%) at 0.1% peptone concentration, while *Bacillus cereus* and *Pseudomonas* sp. showed 73 and 69% decolorization at 0.4 and 0.2% concentration of peptone. Further increasing in concentration, decolorization reduced but biomass was slightly increased (Fig. 7).

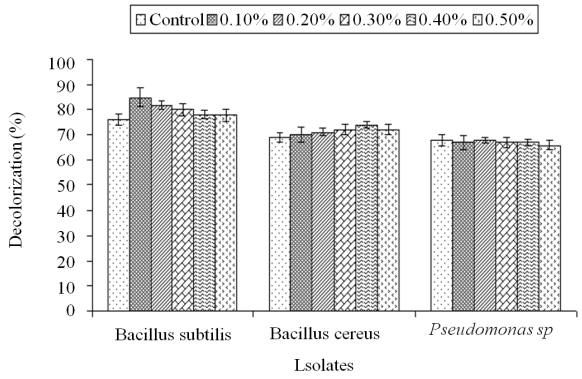

Fig. 7: Effect of different peptone concentration on melanoidin decolorization. The control flask does not contain peptone. Test flasks contained different concentration of peptone in the medium at a level of 0.6% (w/v). Inoculated flasks were incubated at 40°C for 24 h. Error bars presented are mean values of ± standard deviation of triplicates of three independent experiments

DISCUSSION

Among the three bacterial strains *Bacillus subtilis* was found better than *Bacillus cereus* and *Pseudomonas sp*. Melanoidin decolorization ability in microorganisms differe from strain to strain. The constitutive and induced nature of specific microbial enzymes could lead to degradate melanoidin at faster rate. Microorganisms have very diversified metabolic process and regulatory mechanisms.

In this investigation, *Bacillus subtilis* could tolerate 35-50°C without affecting exponential growth phase which could mainly responsible for higher melanoidin decolorization. Some workers have reported that the higher biomass attained within 24-48 h with fast decolorization at a temperature range of 25-40°C (Jiranuntipon *et al*., 2008; Ravikumar *et al*., 2011). Cetin and Donmez (2006) observed the suppressed decolorizing activity at 45°C, might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at higher temperature. Some workers reported that melanoidin decolorization by some bacteria was due to its enzymatic activity like oxidase, ligninase and peroxidases. Mohana *et al*. (2007) reported that increasing temperature from 20-37°C affected the decolorization and further increase in temperature above 40°C adversely affected the decolorization ability of bacterial consortium (*Pseudomonas* and *Proteus mirabilis*). Above 45°C, enzyme activity of microorganism was affected like peroxidases. Thus, it may be suggested that the optimal temperature for melanoidin decolorization depend on the variation of microbial strains and their genetic diversity as they have been isolated from a very wide range of climatic conditions.

Some worker were reported that melanoidin decolorization by several microorganisms showed within a short periods of incubation with optimum growth (Sirianuntapiboon *et al*., 2004a; 2004b; Chavan *et al*., 2006; Sanroman *et al*., 2010), while in case of filamentous fungi, it takes longer period (Kim and Shoda, 1999). Submerged condition is favorable for most of the bacteria. Mohana *et al*. (2007) reported that time course of effluent decolorization was studied alongwith the growth of the consortium. During maximum growth, maximum enzyme production was achieved which are responsible for melanoidin decolorization by microorganism. Maximum growth also inhibits melanoidin decolorization due to production of some other enzymes or metabolites by the microorganism as a feedback inhibition mechanism during metabolism (Jadhav *et al*., 2011). In the present investigation, bacterial strains showed maximum decolorization in a very short period i.e. 24-48 h of incubation when compared to fungi.

Melanoidin decolorization was studied at different range of pH (3.0-7.0) by various workers and have reported that enzymes formed by microorganism during the decolorization was effective only in acidic conditions (Seyis and Subasing, 2009). At high pH, increase in the color was due to the polymerization of melanoidins and higher nutrient utilization (Adikane *et al*., 2006; Jiranuntipon *et al*., 2008). However, overall significant decolorization was obtained in the optimal range of 5.0-6.0 which confirms the significant role of pH in color removal. In this investigation, maximum decolorization was recorded at pH 6.0-7.0 by the bacteria. Similar results were reported when soil samples were used as inoculum instead of isolated organisms (Adikane *et al*., 2006; Pazouki *et al*., 2008; Ravikumar *et al*., 2011). Above and below of the optimum pH, melanoidin decolorization reduced due to inhibition of the enzyme production. All enzymes are proteinous in nature, therefore, some proteins denatured at higher or lower pH value. However, microorganism has a specific pH for their growth and enzyme activity.

In this investigation, maximum melanoidin decolorization was observed in glucose as well as fructose as carbon source at the level of 0.5% and even 0.1% glucose showed same decolorization potential. This effect can be explained that during initial phase of growth, organism utilizes easily available carbon sources added to the medium and then starts to degrade spentwash that is complex carbon source (Kumar *et al*.,

1997). Ohmomo *et al*. (1987) reported that glucose was the best carbon source, which utilized by *Aspergillus fumigatus* G-2-6 for maximum degradation of melanoidins and further increase in glucose concentration, increased the mycelial biomass but no change in decolorization level. Watanabe *et al*. (1982) have reported that the enzymatic degradation of melanoidin by *Coriolus sp*. No. 20 having an intracellular enzyme, which required active oxygen molecules and sugars (sorbose as well as glucose) in the reaction mixture, was later identified as sorbose oxidase which oxidize glucose into gluconic acid (Miyata *et al*., 2000; D'souza *et al*., 2006). The decline in melanoidin decolorization encountered with high sugar concentration in the medium is probably due to inhibition effect to the enzyme like lignolytic activity of laccase enzyme and oxidation activity of the peroxidase (Raghukumar and Rivonkar, 2001; Guimaraes *et al*., 2005; Pant *et al*., 2008; Jiranuntipon *et al*., 2008; Zhao *et al*., 2010; Ravikumar *et al*., 2011).

Different nitrogen sources were optimized for melanoidin decolorization. Among different nitrogen sources (organic and inorganic), the highest melanoidin decolorization was reported with peptone at the level of 0.1%. Similarly various nitrogen sources were optimized by different workers for melanoidin decolorization, but peptone was the most effective for color removal (Ohmomo *et al*., 1988; Miyata *et al*., 2000; Sirianuntapiboon *et al*., 2004a; 2004b; Ravikumar *et al*., 2011). Kirk *et al*. (1978) reported that enzymatic systems catalyse degradation of lignin and lignin-like compound during the secondary phase of the metabolic growth in the presence of peptone. Synthesis and secretion of lignin peroxidase or ligninase (LiP) and manganese-dependent peroxidase (MnP) are triggered by nutrient limitations such as carbon and nitrogen sources. At high concentration, there was no significant decolorization due to surplus supplementation of nitrogen which inhibited the growth. Similar effect was observed when low concentration of peptone was used as nitrogen source for decolorization of melanoidin pigment present in the spent wash.

CONCLUSION

The thermotolerant strain of *Bacillus subtilis* has ability to decolorized melanoidin at wide range of temperature and pH in the presence of little amount of carbon and nitrogen sources within a very short incubation period, therefore, is beneficial at industrial level for treatment of distillery effluent at economical level.

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