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Distillery Spentwash Decolorization by a Noval Consortium of *Pediococcus acidilactici* and *Candida tropicalis* under Static Condition

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Abstract: The aim of this study was to isolate a consortium of bacterium and yeast from natural resources for better decolorization of distillery spentwash. Consortium exhibited 82±1.5% decolorization within 24 h when incubated at 45°C under static condition in effluent supplemented with 0.1%, glucose; 0.1%, peptone; 0.05%, $MgSO_4$; 0.01%. The cultures were identified as *Pediococcus acidilactici* by 16S rDNA analysis and *Candida tropicalis* on the basis of phenotypic level. It is the first time when thermotolerant melanoidin decolorizing consortium (*Pediococcus acidilactici* and *Candida tropicalis*) isolated from distillery soil was capable to decolorizing melanoidin pigment of distillery effluent. Hence, it was observed that consortium has the ability to degrade the spentwash efficiently. This study could be an approach towards control of ecological pollution and health hazards of humans in and about the distillery location.

Key words: Decolorization, *Pediococcus acidilactici*, *Candida tropicalis*, consortium, melanoidin, thermotolerant

INTRODUCTION

In all over the world wastewater from molasses based alcohol distilleries is known as spentwash containing high BOD and COD. Distillery spentwash contains a dark brown color recalcitrant pigment called melanoidin. An empirical formula of melanoidin is $C_{17-18}H_{26-27}O_{10}N$ with 5000-40,000 Da molecular weight. Melanoidin is a natural browning organic polymer produced by Maillard reaction between amino and carbonyl groups of organic matters and is intimately correlated to humic materials in the natural ecosystem (Aoshima *et al*., 1985; Fujita *et al*., 2000; Samarghandi *et al*., 2007). Melanoidin is toxic such as phenol, so when released in aquatic system, leads to decrease the sunlight diffusion in water bodies (rivers and lakes) and thus hindering the photosynthetic process. Disposal on ground cause decline in soil alkalinity, manganese accessibility and hinders seed germination (Agrawal and Pandey, 1994; Fitzgibbon *et al*., 1995; Jun *et al*., 2009). The dark color of melanoidin remains as a trouble which needs a pretreatment prior to its secure disposal into the ecosystem.

There are several physico-chemical methods have been used for removal of melanoidin from wastewater (Migo *et al*., 1993). However, these processes still have disadvantages due to the high operation cost, high consumption of chemical agents, formation of hazardous by-products and intensive energy requirements. Natural treatments with microbial decolorization processes are drawing attention because they are ecological friendly and cost effective alternatives to chemical decay methods (Moosvi *et al*., 2005). Biological treatments, using fungi such as *Coriolus* sp. (Aoshima *et al*., 1985), *Aspergillus* sp. (Ohmomo *et al*., 1987), *Aspergillus niger* (Miranda *et al*., 1996), *Trametes versicolor* (Benito *et al*., 1997), *Phanerochaete* sp. (Fahy *et al*., 1997), *Phanerochaete chrysosporium* (Kumar *et al*., 1998), *Coriolus versicolor*, *Coriolus hirsutus* (Miyata *et al*., 2000) and *Penicillium decumbes* (Jimenez *et al*., 2003), yeasts such as *Citeromyces* sp. (Sirianuntapiboon *et al*., 2004b) and certain bacteria such as *Bacillus* sp. (Murata *et al*., 1992; Kumar and Chandra, 2006; Rico *et al*., 1997), *Pseudomonas fluorescence* (Dahiya *et al*., 2001) and acetogenic bacteria (Sirianuntapiboon *et al*., 2004a) have been reported.

There are several reports for melanoidin decolorization by filamentous fungi, which adsorption melanoidin in their mycelial system as well as also decolorized it through ligninolytic enzymes (Watanabe *et al*., 1982). However, the long growth cycle and huge spore formation limit the use of the filamentous fungi for decolorization at commercial level due to aeroallergenic spores spreading in the air, while yeast does not create such problems and also grow well in

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aquatic system. Therefore, bacterial and yeast strains are promising for higher decolorization due to its faster growth rate and also co-exist without any hindrance in metabolic functions of individual culture (Adikane *et al*., 2006; Kumar and Chandra, 2006; Jiranuntipon *et al*., 2008). The consortium of *Bacillus* spp. showed a two-to fourfold increase in melanoidin decolorization over that exhibited by any single *Bacillus* isolate (Kumar and Chandra, 2006; Jiranuntipon *et al*., 2008) also reported synthetic melanoidin decolorization by bacterial consortium (*Klebsiella oxytoca*, *Serratia mercescens*, *Citrobacter* sp. and unknown bacterium).

Keeping the above in view, the present study was aimed at isolation, identification and development of a thermotolerant consortium of bacterium and yeast isolate from soil of distillery effluent. Decolorization efficiency in different medium with different ratio of inoculum size of bacterium and yeast for consortium development was investigated. The effect of various temperature and incubation period was also studies for maximum decolorization under static condition.

MATERIALS AND METHODS

Distillery spentwash (DSW): The molasses spentwash was collected aseptically from Masuadha sugarcane distillery, faizabad (U.P.), 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 analysis of different physico-chemical parameters like color, odor, pH, biological oxygen demand (BOD), Chemical Oxygen Demand (COD), Total Dissolved Solids (TDS), sulphates, phosphorous and chlorine were analyzed as per the standard methods of APHA (1995).

Isolation, screening and identification of melanoidin decolorizing bacteria and yeast for consortium development: The soil samples were collected in sterile polyethylene bags from adjoining areas of Masaudha Distillery, Faizabad (U.P.) India for isolation. The soil (1.0 g) sample was serially diluted upto 10^{-6} to 10^{-7} and 0.1 mL was spreaded over basal agar plates (pH 6.0) containing, 0.2%, K_2HPO_4, 0.1%, KH_2PO_4, 0.01% $MgSO_4.12H_2O$, 0.5% glucose and 0.1% yeast extract with centrifuged (10,000 rpm for 10 min) distillery spentwash (3.5 OD) diluted with sterile distilled water. The inoculated plates were subsequently incubated for 24-48 h at 35±2 and 45±2°C for isolation of bacteria and yeast, respectively. Thereafter, the selection of efficient isolates for potential decolorization was done on the basis of qualitative and quantitative estimation. Selected efficient culture of bacterium was characterized first by morphological and biochemical studies as per the Bergey's Manual of Systematic Bacteriology (Holt, 1984). Similarly, selected efficient culture of yeast was also characterized first by morphological and biochemical studies like germ tube test, urea hydrolysis, acid production from carbohydrates, assimilation test, growth on different temperature and growth on Cycloheximide as per standard identification manuals (Kreger-Van Rij, 1984). The identity of bacteria was authenticated from Institute of Microbial Technology (IMTECH), Chandigarh, India based on the phenotypic and genotypic (16S r DNA) level but in case of yeast only at phenotypic level identification was done from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Qualitative and quantitative screening: For qualitative screening the isolated bacterial and yeast cultures were spot inoculated on basal agar plates and incubated at 35±2 and 45±2°C for 24-48 h. The colonies of bacteria and yeast showing significant decolorization, as evidenced by clearing of melanoidin pigment around the colonies were selected for quantitative screening to find most efficient culture. The isolates were maintained on the same medium at 4°C in slants and sub-cultured after every two weeks.

For Quantitative screening, melanoidin decolorizing bacterial and yeast isolates were inoculated in the basal broth medium and incubated at optimum temperature (45°C) and pH (6.0) for maximum decolorization; then broth was centrifuged at 10,000 rpm for 10 min. The supernatant of the centrifuged sample was read at absorbance maximum (A_{475}) using spectrophotometer (Ohmomo *et al*., 1988). The decolorization yield was expressed as decrease in the absorbance at 475 nm against initial absorbance at the same wavelength. Uninoculated medium was served as control. The entire assay was performed in triplicate. The decolorization efficiency of the isolate was calculated as per following equation:

$$\text{Decolorization (\%)} = \frac{I-F}{I}$$

where, I is initial absorbance (Control) and F is absorbance of decolorized medium broth.

Inoculum preparation: Mother culture was prepared by inoculating one full loop of 24 h grown culture on basal agar plate in 50 mL basal broth and incubated at 35°C for 24 h to achieve active exponential phase containing of 50×10^6 CFU mL^{-1}. Suitable amount (0.5%, v/v) of this cell suspension was used to inoculate the test flasks.

Biomass determination: Bacterial and yeast cells in broth were collected by centrifugation (10,000 rpm for 10 min at 4°C), washed with distilled water and dried in an oven at 80°C until getting a constant dried weight reported in the form of dry cell mass (g L^{-1}).

Different parameters for selection of efficient bacterium and yeast for development of suitable consortium (bacterium and yeast): Different parameters were employed for selection of efficient bacterium and yeast strain which used for consortium development. The various process parameters influencing melanoidin decolorization were optimized individually and independently of the others, therefore, the optimized conditions were subsequently used in all the experiments in sequential order. For optimization, the medium was inoculation and incubated at different temperature viz. 25, 30, 35, 40, 45, 50, 55 and 60°C under static condition to find out the optimum temperature. The samples were with drawn at every 8 h interval upto 48 h to study the effect of incubation period. Initial pH also plays an important role in melanoidin decolorization, so pH of medium was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 using either sterile 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH after medium sterilization.

Development of consortium for melanoidin decolorization: For construction of the active bacterial and yeast consortium, different experiments were carried out for selection of efficient bacteria and yeast previously discuss. After selection a loopful culture of efficient bacterial and yeast from basal agar plate was precultured in 50 mL basal broth at 45°C under static condition. After 24 h, the bacterial and yeast cells of both strain were harvested by centrifugation at 10,000 rpm at 4°C for 10 min then washed with double distilled water. Washed bacterial and yeast cells at appropriate volume (0.5%, v/v) were subsequently inoculated into fresh melanoidin containing basal broth medium to obtain an initial OD_{620} of 0.5. This consortium was incubated at 45°C for 24 h under static condition. All assays were performed in triplicate and compared with respective uninoculated control.

Selection of efficient medium for melanoidin decolorization: An experiment was conducted to select a suitable medium for efficient decolorization by bacterium, yeast and consortium. The medium having various combinations of glucose, peptone and effluent was used to evaluate decolorization potential of the isolates. Three types of media with different composition were used to evaluate.

Medium A: Effluent without carbon and nitrogen supplemented medium with 3.5 OD.

Medium B: (Glucose yeast extract peptone effluent medium): 0.1%., glucose, 0.2%., yeast extract, 0.1%., peptone, 0.05%., $MgSO_4$, 0.05%., K_2HPO_4 with 3.5 OD effluent.

Medium C: (Glucose peptone effluent medium): 0.6%., glucose, 0.5%., peptone, 0.05%., $MnSO_4$, 0.05%., K_2HPO_4 with 3.5 OD effluent, respectively.

Optimization of different parameters for melanoidin decolorization by active consortium

Effect of static and shaking condition on the melanoidin decolorization: In order to determine the effect of shaking and static conditions on melanoidin decolorization by bacterium, yeast and consortium were evaluated. In this experiment, medium was supplemented with 0.5% (v/v) inoculum of bacterium, yeast and consortium separately, which incubated at different shaking condition (20 to 140 rpm, at the of interval 20 rpm) in orbital shaker (New Brurswick, USA) and under static condition (served as control) for 24 h at 45°C for better melanoidin decolorization.

Effect of different ratio of bacterium and yeast in consortium for melanoidin decolorization: This study was carried out to verify the effect of different inoculum ratio (1:1, 1:2 and 2:1) of bacterial and yeast strain for active consortium development which applied for effective melanoidin degradation. Different inoculum ratio (1:1, 1:2 and 2:1) of bacterial and yeast cells with 0.5% (v/v) inoculum having 50×10^6 CFU mL^{-1} population were supplemented into 250 mL Erlenmeyer flask containing 50 mL of each melanoidin containing basal broth medium for melanoidin decolorization by effective consortium. The flasks were placed under static condition at 45°C for different incubation periods viz. 8, 16, 24, 32, 40 and 48 h to observe the effect of these ratios on decolorization and biomass production.

Effect of different temperature on melanoidin decolorization by the consortium: In another set of experiment, melanoidin decolorization by bacterium, yeast and consortium were monitored at three different set of temperatures (25, 35 and 45°C) at different incubation periods with optimal condition of different parameters. In this experiment, medium was supplemented with 0.5% (v/v) inoculum of bacteria, yeast and consortium separately, which incubated at three different set of temperature at different incubation periods (8, 16, 24, 32, 40 and 48 h) for achieve higher decolorization.

Statistical analysis: Each experiment was performed twice, each in triplicate and standard deviation for each perimental result was calculated using the Microsoft Excel.

RESULTS

Physico-chemical analysis of distillery effluent: The distillery effluent was analyzed for physico-chemical properties and it was found that the effluent was slightly acidic (pH 4.2) having molasses like odor and dark-brown in color (Table 1). The level of Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Dissolved Oxygen (DO), Total Dissolved Solid (TDS), Total Suspended Solid (TSS), Total Nitrogen (TN), phosphorus, potassium, calcium and sulphate were well above the permissible limits (Indian Standards Institution, ISI, 1977). Only sodium is present in significant quantities.

Isolation, screening and identification of melanoidin decolorizing bacteria and yeast for consortium development: Fifty eight bacterial and 24 yeast cultures were isolated from soil sample, among which 10 bacteria and 8 yeast were found thermotolerant as well as mesophilic decolorizer as they exhibited clear zone diameter >1.0 cm on melanoidin containing basal agar plates (pH 6.0) after 24-48 h of incubation at 35-45°C. The isolates exhibited >1.0 cm of clear zone diameter around the colony were considered as effective decolorizers.

During quantitative screening, ten isolates of bacteria and eight isolates of yeast were inoculated individually in 50 mL of basal broth medium (pH 6.0) containing molasses wastewater at 35 and 45°C for 24-48 h for selection of efficient thermotolerant melanoidin decolorizing bacterium and yeast. Of the ten efficient bacterial isolates, B-25, B-44 and B-53 strains was found the potent melanoidin decolorizer but maximum 68% at 35°C and 70% at 45°C decolorization was showed by bacterial strain B-25 within 24 h. Similarly, out of the eight efficient yeast isolates, RG-9, RG-12 and RG-20 strains was found effective for decolorization but RG-9 showed maximum 63% at 35°C and 67% at 45°C decolorization within 24 h (Table 2). The capability to grow at 35 and 45°C reveals mesophilic as well as thermotolerant nature of the isolates.

On account of morphological and biochemical characteristics, bacterial strain B-25 and yeast strain RG-9 were identified as *Pediococcus* sp. and *Candida tropicalis* (Table 3) by IMTECH, Chandigarh (India). Analysis of 16S rDNA sequence (1426 bp) revealed strain B-25, 99.5% (MTCC, Chandigarh) homology with *Pediococcus acidilactici* strains and was designated as *Pediococcus acidilactici*

Table 1: Physico-chemical properties of distillery effluent (spentwash)

Parameters (mg L^{-1})	Value of distillery effluent	Permissible limit
Color	Dark brown	-
Odour	Like molasses	-
Temperature °C	82	40-45
pH	4.2	5.5-8.8
Total dissolved solid (mg L^{-1})	81733	2100
Total suspended solid (mg L^{-1})	5933	100
Dissolved oxygen (mg L^{-1})	0	5.0
Biological oxygen demand (mg L^{-1})	46666	30-100
Chemical oxygen demand (mg L^{-1})	104130	250
Total nitrogen (mg L^{-1})	1635	10
Phosphorus (mg L^{-1})	163	2
Potassium (mg L^{-1})	8766	-
Sodium (mg L^{-1})	211	600
Calcium (mg L^{-1})	1816	75
Sulphate (mg L^{-1})	1738	200

Table 2: Selection of efficient bacterium and yeast isolate

	Decolorization (%)			Decolorization (%)	
Bacterial strains	Temperature (35°C)	Temperature (45°C)	Yeast strains	Temperature (35°C)	Temperature (45°C)
B-5	47±1.5	43±1.0	RG-4	34±1.9	40±1.9
B-12	50±1.5	45±1.5	RG-7	30±1.8	35±1.8
B-18	43±1.2	50±1.2	RG-9	63±2.1	67±2.1
B-23	47±1.6	50±1.6	RG-12	52±1.7	57±1.7
B-25	68±2.0	70±2.0	RG-15	29±1.0	36±1.0
B-30	45±1.0	43±1.0	RG-19	30±2.3	39±2.3
B-38	50±1.2	45±1.2	RG-20	43±1.3	47±1.3
B-44	53±1.0	50±1.0	RG-22	38±1.0	41±1.0
B-47	41±1.5	45±1.5			
B-53	60±2.0	55±2.0			

B: Bacterial isolates (Bacterial cultures are designated by the B and No. are the isolates no provide by us during isolation, For example B-25 means Bacterium 25 in which B used for bacteria and 25 is bacteria No. 25), RG: Yeast Isolated (Yeast cultures are designated by the RG and No. are the isolates no provide by us during isolation For example RG-9 means Yeast-9 in which RG used for Yeast and 9 is yeast No. 9)

Table 3: Morphological, physiological and biochemical characteristics of the selected RG-9 isolate

Characteristics	Results	Characteristics	Results
Morphological tests		**Assimilation tests (Hydrolysis)**	
Shape	Spherical	D-mannitol	+
Surface	Smooth	Succinic acid	+
Size (μm)	3.0-5.5×4.0-9.0	Soluble starch	+
Color	White	D-xylose	+
Physiological test		Trehalose	+
Germ tube test	-	Maltose	+
Hydrolysis of urea	-	Galactose	+
Growth on	+	Potassium nitrate	-
Cycloheximide medium			
Growth at 37-45°C	+	D-arabinose	-
Biochemical test		Inositol	-
Gas production from		Erythritol	-
Glucose	+	Galactitol	-
Maltose	+	Melibiose	-
Galactose	+	Raffinose	-
Trehalose	+	Lactose	-
Sucrose	+	L-rhamnose	-
Lactose	+	Capsules	-
Assimilation tests (Hydrolysis)		**Growth appearance on cornmeal and tween 80 agar**	
Glucose	+	Branched pseudohyphae	Present
D-glucitol	+	Terminal vesicles	Absent
L- arabinose	+	Ovoid blastoconidia	Present

+: Positive, -: Negative

B-25. The 1426bp 16S rDNA sequence was submitted to Genbank [JQ: 387601]. The strain B-25 was in the same cluster of phylogenetic tree (Fig. 1) with different strains of *Pediococcus acidilactici*.

Table 4: Different parameters for selection of efficient bacterium and yeast for consortium development

	Decolorization (%)					
Parameters	B-25	B-44	B-53	RG-9	RG-12	RG-20
Temperature (°C)						
25	26	23	19	55	49	37
30	49	44	39	60	54	42
35	76	61	53	68	55	45
40	76	59	51	72	58	47
45	76	58	50	71	58	49
50	75	57	50	71	58	49
Incubation period (h)						
8	39	12	18	35	28	26
16	51	23	28	55	38	33
24	76	34	48	72	53	48
32	76	38	50	70	52	47
40	76	40	52	70	52	47
48	75	55	52	70	52	47
pH						
4.0	28	23	21	40	23	34
4.5	49	40	38	52	39	40
5.0	69	49	41	69	41	46
5.5	76	57	45	72	58	48
6.0	78	62	54	72	60	50
6.5	76	62	54	71	59	50
7.0	70	55	48	71	58	50

B: Bacterial isolates (Bacterial cultures are designated by the B and No. are the isolates no provide by us during isolation), RG: Yeast Isolated (Yeast cultures are designated by the RG and No. are the isolates No. provide by us during isolation)

Optimization of different physico-chemical and nutritional parameters for selection of efficient strain for consortium development: From above experimental results, three efficient bacterial and three efficient yeast strain were selected for optimization of different parameters (temperature, pH and incubation periods) for maximum decolorization and select best strain for consortium development. In this experiment our strain B-25 and RG-9 showed maximum melanoidin decolorization (76 and 72%) at 45°C while other bacteria and yeast strains showed less decolorization at different temperature (Table 4). Further increase in temperature could not affect the decolorization efficiency by bacterial strain B-25 and yeast strain RG-9.

Incubation period is another crucial factor affecting melanoidin decolorization. Optimal time for maximum decolorization (76 and 72%) by bacterium B-25 and yeast RG-9 was 24 h while other strains of bacteria and yeast take more time for better decolorization. Further increase in the incubation period did not increase the decolorization (Table 4).

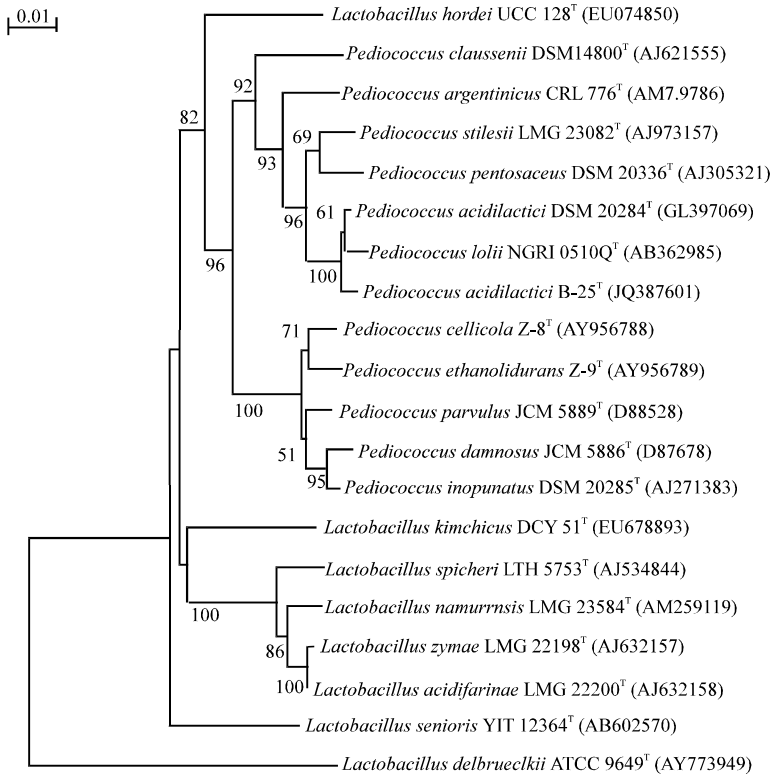

Fig. 1: Phylogenetic tree showing relation between strain B-25 and other *Pediococcus* strains

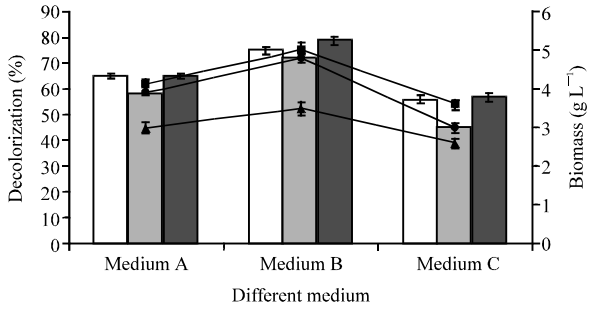

Fig. 2: Selection of medium for better melanoidin decolorization, Decolorization yield (□, ▨, ■) and Biomass production (—▲—, —■—, —●—) by bacteria, yeast and consortium. The test flasks were inoculated individually with bacterium, yeast and consortium and incubated at 45°C for 24 h under static condition. Error bars presented are mean values of±Standard deviation of triplicates of three independent experiments. Compositions of three different media are given below, Medium A (WWE): Effluent without carbon and nitrogen supplemented medium with 3.5 OD, Medium B (GYPE): 0.1%., glucose, 0.2%., yeast extract, 0.1%., peptone, 0.05%., $MgSO_4$, 0.05%., K_2HPO_4 with 3.5 OD effluent, Medium C (GPE): 0.6%., glucose, 0.5%., peptone, 0.05%., $MnSO_4$, 0.05%., K_2HPO_4 with 3.5 OD effluent, respectively

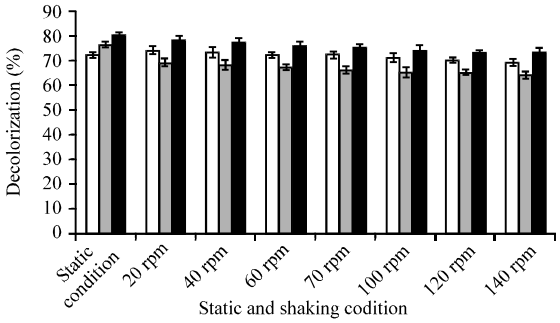

Fig. 3: Effect of shaking and static condition on melanoidin decolorization, Decolorization yield by bacteria, yeast and consortium (□, ▨, ■). The test flasks were inoculated individually with bacterium, yeast and consortium and incubated at 45°C for 24 h at pH 6.0. Test flasks kept under static condition were served as control. Error bars presented are mean values of ±standard deviation of triplicates of three independent experiments

Melanoidin decolorization was critically affected by different pH and showed differing trends. From Table 4 it was observed that maximum (78 and 75%) color removal was obtained at pH 6.0 by bacterium B-25 and at pH 5.5-6.0 by yeast RG-9 while other bacteria and yeast did not showed a significant level of decolorization. Beyond pH 6.0 decrease in melanoidin decolorization was observed.

From these results it cleared that maximum melanoidin decolorization was obtained by bacterium B-25 and yeast RG-9 at different parameters while other strains of bacteria and yeast showed less melanoidin decolorization at same parameters. So on the basis of above observations bacterium B-25 and yeast RG-9 was selected for the developed of consortium to obtain significant melanoidin decolorization.

Development of consortium for melanoidin decolorization: After selection of efficient strain of bacterium B-25 and yeast RG-9, construction of consortium was carried out. In this experiment both culture grow separately in basal broth to obtain its optimum growth, then broth was centrifuged and washed with the distilled water. After that, equal mass of both cultures was inoculated in the fresh melanoidin containing basal broth for better melanoidin decolorization by their combined metabolic activity.

Selection of efficient medium: The effect of medium composition on melanoidin decolorization by bacterium, yeast and consortium are presented in Fig. 2. Bacterium, yeast and their consortium showed maximum 76, 72 and 79% decolorization with 3.5, 4.8 and 5.0 g L^{-1} biomass production in Medium B followed by medium A whereas medium C supported minimum decolorization. The availability of more carbon and nitrogen source in medium B comparison to medium A and C could be the reason for better decolorization. Hence medium B was selected for optimization of physico-chemical and nutritional parameters for maximum melanoidin decolorization by consortium.

Effect of static and shaking conditions on the melanoidin decolorization: Melanoidin decolorization by consortium was affected under shaking and static conditions. In this experiment melanoidin decolorization by consortium was active in all the conditions employed with maximum (80%) decolorization under static condition (Fig. 3). With regard to shaking condition it was observed that the higher shaking did not support decolorization by consortium as increase in rpm resulted in decrease in decolorization. However, at lowest rpm same level of decolorization, as for static condition, was observed. In case of yeast, it also exhibited maximum decolorization under static condition

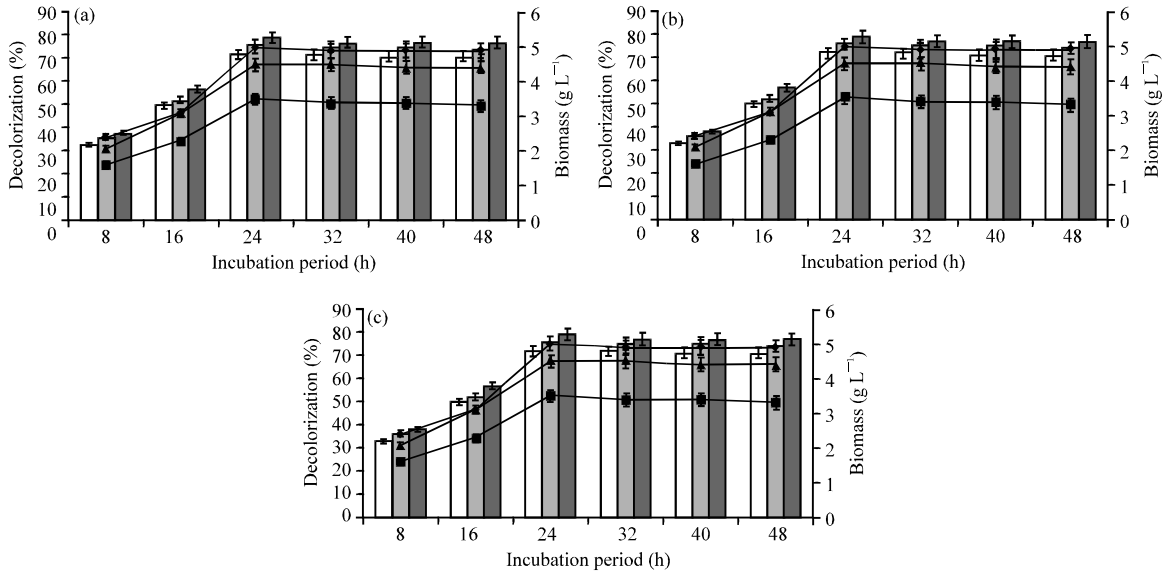

Fig. 4(a-c): Effect of different ratio of inoculum on melanoidin decolorization at different incubation period, Decolorization yield by yeast, bacteria and consortium (□, ■, ■). Biomass production by yeast, bacteria and consortium (—▲—, —■—, —●—). The test flasks were inoculated individually with bacterium, yeast and consortium and incubated at 45°C temperature for 24-48 h at pH 6.0 under static condition. In this experiment test flasks of consortium was inoculated with three different ratio of inoculum of bacterium and yeast (1:1, 1:2 and 2:1) at 45°C for 24-48 h under static condition. Error bars presented are mean values of ±standard deviation of triplicates of three independent experiments

but bacteria showed significant decolorization at shaking as well as under static condition. The remarkable decolorization under the static condition revealed facultative nature of microorganisms. This experiment was conducted to screen the best among the two, shaking and static condition for highest decolorization. Highly aerobic condition did not show significant percentage of melanoidin decolorization as compared to facultative decolorization process; hence facultative decolorization process was adopted for this study. Bacteria, yeast and their consortium are proficient for satisfactory percentage of melanoidin decolorization in facultative anaerobic condition; therefore, it can be exploited for industrial decolorization due to less amount of energy is required.

Effect of different inocolum ratio for melanoidin decolorization: For development of an efficient and stable consortium, different inocoulum ratios (1:1, 1:2 and 2:1) of bacterium and yeast strain was inoculated in the medium and incubated at their optimum physico-chemical conditions. Maximum melanoidin decolorization (80%) was reported by consortium having 1:1 inoculum ratio of bacterium and yeast with in 24 h of incubation while individual culture of bacterium and yeast showed maximum 77 and 67% decolorization under same conditions. Further incubation did not increase the rate of decolorization. Similarly, maximum biomass was recorded in 24 h in case of bacterium (3.5 g L^{-1}), yeast (4.7 g L^{-1}) and consortium (5.0 g L^{-1}). However, consortium produced higher biomass as compared to individual effect of bacteria and yeast (Fig. 4a).

In another set of experiment, 1:2 inoculum ratio was tested for melanoidin decolorization which showed maximum 80% decolorization similar to 1:1 ratio of inoculum size result (Fig. 4b). In the third set of experiment where 2:1 inoculum ratio was used for consortium development showed almost same trend of melanoidin decolorization and biomass production as reported in case of 1:1 and 1:2 inoculum ratio (Fig. 4c). So it can be concluded from above observations that melanoidin decolorization was not affected by inoculum size.

Effect of temperature on melanoidin decolorization: Mix culture of microorganisms are generally affected by temperature, therefore, an experiment was also conducted to evaluate such effect on the consortium for melanoidin decolorization. Melanoidin decolorization by consortium was active at all temperatures employed with maximium decolorization at 45°C in 24 h of incubation. It exhibited 82% decolorization with 5.0 g L^{-1} biomass production at

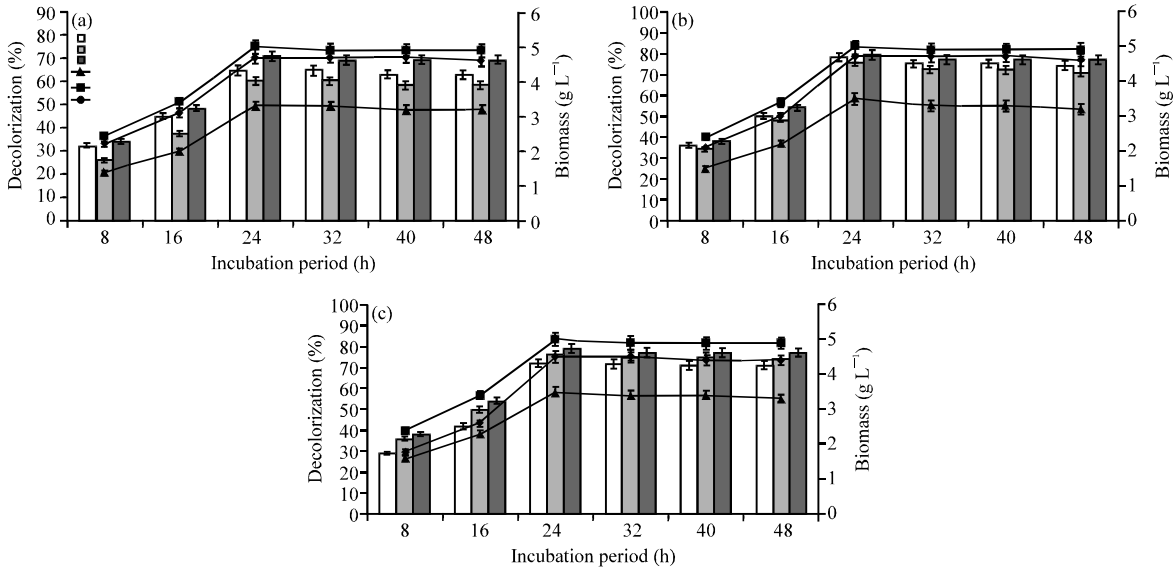

Fig. 5(a-c): Effect of different temperature and incubation period on melanoidin decolorization, Decolorization yield by yeast, bacteria and consortium (□, ▫, ■). Biomass production by yeast, bacteria and consortium (—▲—, —■—, —●—). The test flasks were inoculated individually with bacterium, yeast and consortium and incubated at three different temperature (25, 35 and 45°C) for 24-48 h at pH 6.0 under static condition. Test flasks of consortium were inoculated with 1:1 ratios of bacterium and yeast. Error bars presented are mean values of ±standard deviation of triplicates of three independent experiments

45°C. Even at 25 and 35°C, it exhibited 78 and 79% decolorization, respectively (Fig. 5a, b and c). The remarkable decolorization in the temperature range 25-45°C revealed mesophilic as well as thermotolerant nature of consortium. In case of bacteria and yeast, bacterial strain showed maximum decolorization at 45°C and yeast strain showed its maximum activity at 25-45°C within 24 h of incubation (Fig. 5a, b and c). From this study it cleared that combined metabolic activity of consortium is better for melanoidin decolorization then individual effect of bacterium and yeast. Further, increase in temperature could not affect the biomass production as well as decolorization efficiency by consortium.

DISCUSSION

In the present study, different physico-chemical analysis of distillery spent wash was carried out for better melanoidin decolorization by consortium. Fifty eight bacterial and 24 yeast cultures were isolated from soil sample, among which 10 bacteria and 8 yeast were found thermotolerant as well as mesophilic decolorizer as they exhibited clear zone diameter >1.0 cm on melanoidin containing basal agar plates (pH 6.0) after 24-48 h of incubation at 35-45°C.

During quantitative screening, out the ten efficient bacterial isolates, B-25, B-44 and B-53 strains was found the potent melanoidin decolorizer but maximum 68% at 35°C and 70% at 45°C decolorization was showed by bacterial strain B-25 within 24 h. Similarly, out of the eight efficient yeast isolates, RG-9, RG-12 and RG-20 strains was found effective for decolorization but RG-9 showed maximum 63% at 35°C and 67% at 45°C decolorization within 24 h (Table 2). The capability to grow at 35 and 45°C reveals mesophilic as well as thermotolerant nature of the isolates.

On account of morphological and biochemical characteristics, bacterial strain B-25 and yeast strain RG-9 were identified as *Pediococcus* sp. and *Candida* sp. by IMTECH, Chandigarh (India). Analysis of 16S rDNA sequence (1426 bp) revealed strain B-25, 99.5% homology with *Pediococcus acidilactici* strains and was designated as *Pediococcus acidilactici* B-25. The 1426 bp 16S rDNA sequence was submitted to Genbank [JQ: 387601]. The strain B-25 was in the same cluster of phylogenetic tree (Fig. 1) with different strains of *Pediococcus acidilactici.* However, the 16S rDNA sequence analysis indicates that it is a different and novel strain of *Pediococcus acidilactici*. In case of efficient yeast strain, RG-9 was identified as *Candida tropicalis* RG-9 at biochemical level from MTCC Chandigarh (Table 3).

For better result, selected efficient cultures of bacteria and yeast were applied for optimization of different parameters (temperature, pH and incubation periods) for decolorization. Thereafter, a prominent consortium was developed from selected efficient stain of bacterium and yeast for better decolorization. It has been reported that temperature is an important factor for melanoidin decolorization (Kumar *et al*., 1997). In this experiment, bacterium B-25 and yeast RG-9 showed 76 and 72% melanoidin decolorization at 45°C while other bacteria and yeast showed less decolorization at different temperature (Table 4). Strains B-25 and RG-9 showed better decolorization potential than other researchers (Kambe *et al*., 1999; Sirianuntapiboon *et al*., 2004a; Tondee *et al*., 2008). Further increase in temperature could not affect the decolorization efficiency by bacterial strain B-25 and yeast strain RG-9. According to Cetin and Donmez (2006) high temperature may result failure in cell viability or inactivation of the enzymes responsible for melanoidin decolorization resulted into concealed decolorizing activity. In contrast, some workers have been reported maximum decolorization at lower temperature range of 25 to 40°C (Chavan *et al*., 2006; Jiranuntipon *et al*., 2008).

Incubation period also influence melanoidin decolorization. Bacterium B-25 and yeast RG-9 showed maximum (76 and 72%) melanoidin decolorization within 24 h while other strains of bacteria and yeast showed lesser decolorization. After 24 h of incubation bacterial cultures showed lesser melanoidin decolorization (Table 4). On contrary, Sirianuntapiboon (1999) reported 90.0 and 96.75% decolorization by acetogenic bacterial strain No.13A and No. BP 103, after 4 days of incubation. Similarly, Tondee *et al*. (2008) reported 60% decolorization by *Issatchenkia orientalis* but after 7 days of incubation. In another study, Chavan *et al*. (2006) and Sirianuntapiboon *et al*. (2004b) have reported a maximum (56 and 68%) decolorization by using *Pseudomonas* sp. and *Citeromyces* sp. WR-43-6 after 3 and 7 days of incubation.

Melanoidin decolorization was significantly affected by different pH and showed differing trends in case of bacteria and yeast. From Table 4 it was observed that maximum (78 and 75%) melanoidin decolorization was obtained at pH 6.0 by bacterium B-25 and at pH 5.5-6.0 by yeast RG-9 while other bacteria and yeast did not showed a significant level of decolorization. Beyond pH 6.0 decrease in melanoidin decolorization was observed. Sirianuntapiboon *et al*. (2004a) have also reported highest decolorizing activity by an acetogenic strain in the pH range of 5.0-7.0. Several other researchers have also reported that maximum melanoidin decolorization was obtained at 5.0-6.0 optimum pH range from yeast strains (Sirianuntapiboon *et al*., 2004b; Tondee *et al*., 2008). Adikane *et al*. (2006) described the crucial role of pH on melanoidins decolorization. The increase in medium pH raises the color intensity of spentwash due to the polymerization of melanoidins which resulted into decrease in microbial decolorization.

From above experimental results it cleared that maximum melanoidin decolorization was obtained by bacterium B-25 and yeast RG-9 at different parameters while other strains of bacteria and yeast showed poor melanoidin decolorization at same parameters. So on the basis of above observations bacterium B-25 and yeast RG-9 was selected for the developed of consortium to obtain significant melanoidin decolorization.

After consortium development, a comparative study on the melanoidin decolorization by bacterium B-25, Yeast RG-9 and their consortium was carried out under different parameters. First effect of medium composition on melanoidin decolorization by bacterium, yeast and consortium was studied presented in Fig. 2. Bacterium, yeast and consortium showed maximum 76, 72 and 79% decolorization with 3.5, 4.8 and 5.0 g L^{-1} biomass production in medium B followed by medium A whereas medium C supported minimum decolorization. The availability of more carbon and nitrogen source in medium B comparison to medium A and C could be the reason for better decolorization. Mohana *et al*. (2007) also optimized different media for better melanoidin decolorization and COD reduction and observed that maximum decolorization (67%) and COD reduction (51%) was obtained from glucose and salt containing medium. Supplementation of a readily available carbon source such as glucose appeared to be necessary for decolorization. Hence medium B was selected for further study for maximum melanoidin decolorization by consortium.

After selection of suitable medium, effect of shaking and static condition on melanoidin decolorization was also carried out. Melanoidin decolorization by consortium was active in all the conditions employed with maximum 80% decolorization under static condition (Fig. 3). With regard to shaking condition it was observed that the higher shaking did not support decolorization by consortium as increase in rpm resulted in decrease in decolorization. However, at lowest rpm same level of decolorization, as for static condition, was observed. In case of yeast, it also exhibited maximum decolorization under static condition but bacteria showed significant decolorization at shaking as well as under static condition. The remarkable decolorization under the static condition revealed facultative nature of microorganisms. This experiment was conducted to screen the best among the

two, shaking and static condition for highest decolorization. Highly aerobic condition did not show significant percentage of melanoidin decolorization as compared to facultative decolorization process; hence facultative decolorization process was adopted for this study. Similarly Mohana *et al.* (2007) reported that bacterial consortium exhibited maximum decolorizing activity only when incubated under static condition, while agitated cultures although grew well and also resulted higher COD reduction (66%) but less decolorization (38%). Jiranuntipon *et al.* (2008) also stated that the decolorization mechanisms of melanoidins-containing wastewater by bacterial consortium MMP1, might be due to metabolism of bacterial cell under facultative and anaerobic conditions such as fermentation and anaerobic respiration. It is very clear that facultative anaerobic decolorization is always beneficial over aerobic decolorization. Bacteria, yeast and their consortium are proficient for satisfactory percentage of melanoidin decolorization in facultative anaerobic condition; therefore, it can be exploited for industrial decolorization due to less amount of energy is required.

For proficient and stable consortium, different inocoulum ratios (1:1, 1:2 and 2:1) of bacterium and yeast strain were also optimized at optimal conditions. Consortium having 1:1 inoculum ratio of bacterium and yeast showed maximum 80% melanoidin decolorization with 5.0 g L^{-1} biomass production with in 24 h of incubation. While individual culture of bacterium and yeast showed maximum 77 and 67% decolorization with 3.5 and 4.7 g L^{-1} biomass production under sanie conditions. Further incubation did not increase the rate of decolorization.

In another set of experiment, 1:2 inoculum ratio was tested for melanoidin decolorization which showed maximum 80% decolorization similar to 1:1 ratio of inoculum size result (Fig. 4b). In the third set of experiment where 2:1 inoculum ratio was used for consortium development showed almost sanie trend of melanoidin decolorization and biomass production as reported in case of 1:1 and 1:2 inoculum ratio (Fig. 4c). So it can be concluded from above observations that melanoidin decolorization was not affected by inoculum size. Similarly, Tondee *et al.* (2008) also reported that inoculum size did not significantly affect melanoidin decolorization yield by yeast strain no.SF9-246. They observed almost sanie trend of melanoidin decolorization by yeast strain with different inoculum size (5, 10, 15, 20, 25 and 30%). In contrast, Mohana *et al.* (2007) reported maximum decolorization and COD reduction at 15% (v/v) inoculum concentration. Similarly, Sirianuntapiboon *et al.* (2004b) also reported maximum decolorization (68%) in the presence of 10% inoculum size. The inoculum size is some time directly propotional to nutrient present and rate of utilization of substrate and enzyme production responsible for decolorization. From these studies it clears that each strain requires a specific inoculum size for maximum decolorization. Some microorganisms grow fast so require a little inoculum size for initial growth in fresh medium for decolorization. Some microorganisms grow slow and require a large inoculum size for initial growth in the medium for decolorization. So in this study our strains required small inoculum for higher decolorization when compared with others.

Mix culture of microorganisms are generally affected by temperature, therefore, an experiment was also conducted to assess such effect on the consortium for melanoidin decolorization. Melanoidin decolorization by consortium was active at all temperatures employed with maximium 82% decolorization with 5.0 g L^{-1} biomass production at 45°C in 24 h of incubation. Even at 25 and 35°C, it exhibited 78 and 79% decolorization, respectively (Fig. 5a, b and c). The remarkable decolorization in the temperature range 25-45°C revealed mesophilic as well as thermotolerant nature of consortium. In case of bacteria and yeast, bacterial strain showed maximum decolorization at 45°C and yeast strain showed its maximum activity at 25-45°C within 24 h of incubation (Fig. 5a, b and c). From this study it cleared that combined metabolic activity of consortium is better for melanoidin decolorization then individual effect of bacterium and yeast. Consortium showed better decolorization potential than Mohana *et al.* (2007) who reported a maximum of 67% decolorization at 37°C by consortium. Biomass production and decolorization efficiency of the consortium could not affected by increasing the temperature. Cetin and Donmez (2006) reported that high temperature may cause failure in cell feasibility or enzymes inactivation responsible for melanoidin decolorization resulted into suppressed decolorizing activity. Hence, it may be suggested that the favorable temperature for decolorization depend on the strains and their genetic variety.

CONCLUSION

In the present investigation spentwash was decolorized by consortium which was isolated from soil samples of distillery spentwash disposal site. The ability of developed consortium to decolorize the melanoidin effectively could be attributed to the fact the isolates used in consortium were exposed to the spentwash and they might have developed properties to use melanoidin as nutrient source. In addition the increased decolorization

by consortium compared to that shown by bacterium and yeast solely may be due to the enhanced effect of coordinated metabolic interactions on melanoidins. The consortium showed maximum (80%) decolorization at 45°C within 24 h under static condition. It is the first instance when a thermotolerant melanoidin decolorizing consortium (bacterium and yeast) being reported for higher decolorization of distillery effluent. The results of color reduction reveal that the consortium has the ability to decolorize melanoidin present in the spentwash under optimum conditions. Thus application of bacterial and yeast consotium for decolorization of distillery effluent could be a pragmatic approach for waste water treatment of distillery industries.

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