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Decolorization of Distillery Spentwash (Melanoidin) by Immobilized Consortium (Bacterium and Yeast) Cell: Entrapped into Sodium Alginate Bead

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ABSTRACT

Sugarcane distilleries use molasses for ethanol production and generate large volume of effluent containing high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) along with melanoidin pigment. The aim of this study was to isolate potential thermotolerant melanoidin decolorizing bacterium and yeast from natural resources for consortium development and entrapped in suitable matrix for immobilization at large scale spentwash treatment. A total 58 bacteria and 24 yeast were isolated from soil sample of distillery site in which *Pediococcus acidilactici* B-25 and *Candida tropicalis* RG-09 showed higher decolorization. These two strains were used for consortium development and then entrapped in sodium alginate for the wastewater treatment in a continuous column immobilization system. The immobilized consortium cells showed maximum 85% decolorization with the optimized parameters such as 2% (w/v) sodium alginate, 2% (w/v) calcium chloride with 16 h curing time, 5 g alginate beads with 2 mm bead diameter. The immobilized cells of consortium in alginate beads are more efficient for the wastewater treatment and can be reused for eighteen cycles (24×18 = 432 h) without any loss in their activity and 22 cycles with 72% residual activity. Immobilization of consortium cells in continuous column system is better than free culture. Among the immobilized cell bioreactors, no doubt that continuous column immobilization is a novel and efficient one which can be adopted for the treatment of industrial wastewater containing melanoidin compounds and other pollutants. A proper choice of immobilized culture, careful consideration of various design parameters for continuous column immobilization will make treatment process cost effective in the long run.

Key words: Melanoidin, consortium, *Pediococcus acidilactici*, *Candida tropicalis*, continuous column system, immobilization

INTRODUCTION

In all over the world wastewater from molasses based alcohol distilleries is known as spentwash containing high BOD and COD. Spentwash contains about 2% of dark brown recalcitrant pigment called melanoidin which has an empirical formula of $C_{17\text{-}18}H_{26\text{-}27}O_{10}N$ and molecular weight between 5000 and 40,000 Da. Melanoidin is known as a natural browning polymer produced by Maillard reaction between amino and carbonyl groups of organic matters and is closely related to humic substances in the natural environment (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 reduction of sunlight penetration in rivers, lakes and thereby reducing the photosynthetic activity. Disposal on land cause reduction in soil alkalinity, manganese availability and inhibits seed germination (Agrawal and Pandey, 1994; Fitzgibbon *et al.*, 1995; Jun *et al.*, 2009). The dark color remains as a problem which requires a pretreatment before its safe disposal into the environment.

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. Biological treatments with microbial biodegradation /decolorization methods are drawing attention because they are environmental friendly and cost competitive (Moosvi *et al.*, 2005). There are a good number of reports showing the role of fungi in the decolorization of melanoidin by adsorption to mycelia as well as the role of ligninolytic enzymes (Watanabe *et al.*, 1982). However, the long growth cycle and spore formation limit the performance of the fungal system and did not grow well in aquatic system. In contrast, bacterial and yeast strains are promising for decolorization due to its faster growth rate in aquatic system and may require a mixed community to decolorize melanoidin through combined metabolic mode of individual culture (Adikane *et al.*, 2006; Kumar and Chandra, 2006; Jiranuntipon *et al.*, 2008). The mixed culture of *Bacillus* sp. exhibited a two four fold increase in melanoidin decolorization over that showed by any individual *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).

In order to achieve an effective continuous wastewater treatment with consortium, a column system has to be developed in which the consortium cells can grow well while keeping the effluent degrading activity for long periods. There are only few reports specifically on melanoidin-containing wastewater decolorization in bioreactors. Ohmomo *et al.* (1988) reported the continuous decolorization of molasses wastewater in a bubbling column reactor with *Coriolus versicolor* immobilized within Ca-alginate gel. Fujita *et al.* (2000) also reported that decolorization of melanoidin present in an effluent, using a bioreactor with *Coriolus hirsutus* immobilized onto polyurethane foam cubes. Guimaraes *et al.* (2005) reported continuous decolorization of a sugar refinery wastewater in a modified rotating biological contactor with *Phanerochaete chrysosporium* immobilized on polyurethane foam disks.

The aim of this study was to find a suitable industrial biological treatment process for efficient decolorization of distillery effluent. The continuous column system is a traditional technology for large-scale wastewater treatment applications, offering several advantages: low-shear environment, easy scale-up, high surface area per unit volume, low maintenance costs, low energy requirements, simple construction and operation. The use of sodium alginate was found to be an emerging technology for biological wastewater treatment.

MATERIALS AND METHODS

Distillery spent wash (DSW): The molasses spentwash was collected aseptically from Masuadha 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. The stored distillery spentwash was filtered through (Whatman No: 1) filter paper and was diluted with distilled water (Pazouki *et al.*, 2008). The analysis of different physico-chemical parameters like color, odor, pH, Biological Oxygen

Demand (BOD), chemical oxygen demand (COD), total sugars, total dissolved solids (TDS), sulphates, phosphorous and calcium were analyzed as per the standard methods of APHA, AWWA, WPCF (1998).

Isolation, screening and identification of melanoidin decolorizing bacteria and yeast: 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 was characterized first by morphological and biochemical studies as per the Bergey's Manual of Systematic Bacteriology (Krieg and 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 (Krieg and Holt, 1984). The identity of bacteria was authenticated from Institute of Microbial Technology (IMTECH), Chandigarh, India based on the phenotypic and genotypic (16S rDNA) 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: The colonies of bacteria and yeast showing significant decolorization, as evidenced by clearing of melanoidin pigment around the colonies on the basal agar plates were selected and purified by repeated streaking. The cultures were maintained on the same medium at 4°C in slants and sub-cultured after every two weeks.

The melanoidin decolorizing bacterial and yeast isolate was 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 served as control. The entire assay was performed in triplicate. The decolorization efficiency of the isolate was calculated as per following equation:

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

where, I is the Initial absorbance (Control) and F is the Absorbance of decolorized medium broth.

Inoculum preparation: Mother culture of bacteria and yeast was prepared by inoculating one full loop of 24 h grown culture in 50 mL basal broth and then incubated at 45°C for 24 h to achieve active exponential phase of culture for consortium development.

Development of consortium: For development of consortium, active culture of bacteria and yeast grow in basal broth for 24 h for their optimal growth at 45°C. After 24 h of incubation, each culture

was harvested by centrifugation at 10,000 rpm at 4°C for 10 min then washed with double distilled water. Washed culture of both the organism was used, containing 50×10^6 CFU mL^{-1} population, inoculated into fresh melanoidin containing wastewater medium to obtain an initial OD_{600} of 0.5%. Thereafter, the consortium was incubated at optimal temperature under static condition for their optimal growth and then used for immobilization in continuous column system for higher decolorization at industrial level.

Design of the column and its operational parameters: Continuous column system used for immobilization was divided into two part (1) container or reservoir and (2) column. Reservoir contains sterile melanoidin containing medium. The main functioning system of this bioreactor is column, 120 cm long, 5 cm in diameter and lower portion of the column is like a burette (2 mm in diameter) controlled by a regulator, through which elution of the effluent (mL/min) was regulated. Container (3 L) and column (2 L) connected to each other by a thin capillary system with 1mm in diameter and regulated by another regulator which control/set the flow of effluent medium from container to column. The upper portions of the column is close by a bacterial filter membrane and also contain a little aeration tube from which filtered air come in the medium contact (Fig. 1). One-third part of column was filled with sodium alginate bead having consortium cells and sterile melanoidin containing medium. After decolorization of melanoidin, remove the decolorized medium from the column and filled sterile medium from container into column for further decolorization. Removal of 2 L decolorized medium or solution from column in 24 h of incubation considered as one cycle.

Immobilization of consortium: To achieve a simple and cost effective melanoidin decolorization at higher rates, the biomass (50×10^6 CFU mL^{-1}) was immobilized in various polymeric materials such as sodium alginate, agar, agarose and polyacrylamide (2%).

Biomass production: For the production of biomass the microbial consortium were grown in broth (100 ml) at temperature 45°C and pH 6.0 for 24 h. The biomass was harvested by centrifugation at 10,000 rpm for 10 min. The pellet was then washed thoroughly with distilled water to remove the growth medium sticking on its surface. The immobilization of biomass via entrapment with different polymeric materials was carried out as follows:

Sodium alginate: The biomass was immobilized by entrapment in polymer matrix of Na-alginate. Slurry of Na-alginate (2% w/v) was prepared in hot distilled water with constant stirring (Araujo and Teixeira, 1997). After cooling, 50×10^6 cfu mL^{-1} of biomass were added and stirred on magnetic stirrer for even dispersal. The alginate biomass slurry was introduced in to 2% $CaCl_2.2H_2O$ for polymerization and bead formation using 2 mL syringe. The resultant beads were of 2-3 mm diameter. The biomass entrapped beads were cured in this solution for 24 h and then washed twice with distilled water. The curing procedure hardened the beads and resulted in the formation of a favorable micro-porous structure. Finally, the beads were stored at 4°C in distilled water until further use.

Agar: The agar solution (2%) was prepared by dissolving it in distilled water at 90°C (Lopez *et al.*, 1997). The biomass was added at room temperature and evenly dispersed by stirring.

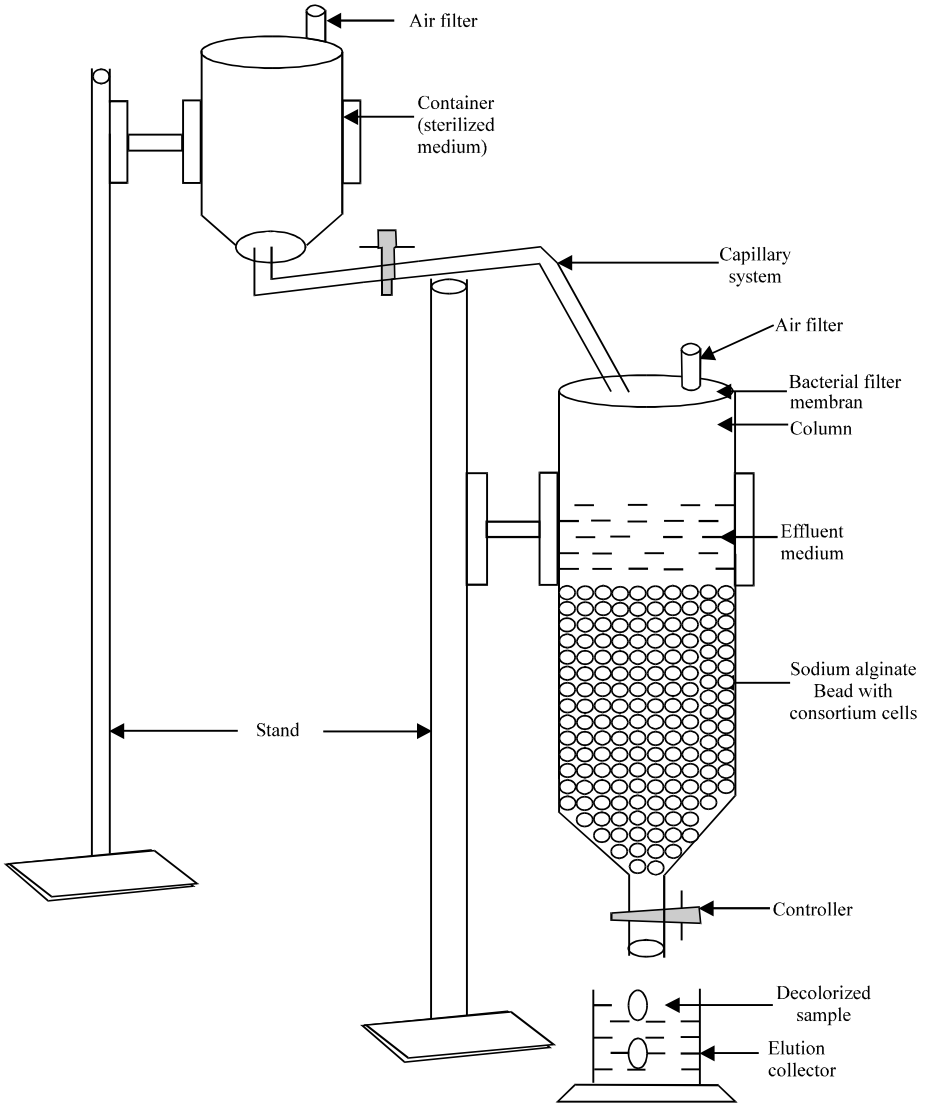

Fig. 1: Immobilization by continuous column system

Spherical beads were obtained on drop wise addition of slurry in to a hydrophobic liquid phase (Sunflower oil, Saffola) over distilled water. The beads were collected and then washed with 0.01% Triton X-100 to eliminate residual oil phase (Nilsson *et al*., 1987).

Agarose: Agarose solution (2%) was prepared by dissolving in distilled water at 90°C, cooling to room temperature (Uchiyama *et al*., 1994). The biomass was evenly distributed in it to make slurry and then rapidly poured in to Petri-plates kept on ice. After solidification on the Petri-plate, 3 mm^3 cubes were cut and washed with distilled water.

Polyacrylamide: A cell suspension was prepared by adding 0.5 g (wet cells) cells to 10 mL chilled sterile distilled water. To another 10 mL of 0.2 M sterile phosphate buffer (pH 6.0), the following chemicals were added: 2.85 g acrylamide, 0.15 g bisacrylamide, 10 mg ammonium-persulphate and

1 mL TEMED (N,N,N1,N1 tetramethylethylenediamine). The cell suspension and the above phosphate buffer mixture was mixed well and poured into sterile Petri plates. After polymerization (solidification), the acrylamide gel was cut into equal size cubes (4 mm^3), transferred to 0.2 M phosphate buffer (pH 6.0) and kept in the refrigerator for 1 h for curing. The cubes were washed 3 to 4 times with sterile distilled water and stored in sterile distilled water at 4°C until use.

Optimization of different parameters for decolorization in continuous column system
Effect of different support material: Different support materials (sodium alginate, agar, polyacrylamide and agrose) were optimized for immobilization of wastewater treatment for higher decolorization at large scale.

Effect of different concentration of sodium alginate: To determine the optimum concentration of sodium alginate for cell immobilization, various concentrations of sodium alginate (1, 1.5, 2, 2.5 and 3%, w/v) were used to prepare alginate beads. The beads were prepared as described earlier in 2% $CaCl_2$ solution, transferred into the newly formulated production and incubated at 45°C for 24 h.

Effect of $Cacl_2$ concentration: The concentration of the cationic solution has a significant effect on the stability and pore size of the bead. Immobilized beads were prepared with the same amount of cells in 2% (w/v) sodium alginate solution using different $CaCl_2$ concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4.0%, w/v). The beads were transferred into the medium and incubated at 45°C for 24 h.

Effect of curing time: The effect of curing time on calcium alginate beads stability and production of enzyme for melanoidin decolorization was tested with varying curing time (8, 16 and 26 h) at 4°C before used.

Effect of bead size: To study the effect of bead size on melanoidin decolorization, three different sizes of alginate beads (2, 4 and 6 mm) were prepared by the different diameters of syringe. Equal cell mass containing beads were transferred into column and fermentations were conducted.

Effect of beads weights: To optimize the bead weight for a particular fixed concentration of substrate, an experiment was carried out. Different amount of beads viz 5, 10, 15 and 20 g beads was packed in immobilization column unit. Inoculums size plays an important role in immobihzation for melanoidin decolorization.

Reusability of gel matrix (repeated-batch): One of the advantages of using immobilized cells is that they can be used repeatedly. Therefore, the reusability of immobilized consortium cells was examined. After attaining the maximum melanoidin decolorization (24 h), the decolorized solution eluted out from the column and beads were used for the next use by adding a fresh production medium (2 L). The process was repeated for several batches until the melanoidin decolorization was decreased. The number of cycle's viz., 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 were calculated on the basis of the capacity of the main column which releases the 2 liter treated effluent, amount was considered as one cycle.

Statistical analysis: All the experiments were carried out in triplicate and the data were analyzed a standard deviation level.

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. Only sodium is present in significant quantities.

Isolation, screening and identification of melanoidin decolorizing bacteria and yeast: Fifty eight (58) bacterial and twenty four (24) yeast cultures were isolated from soil sample, among which ten (10) bacteria and eight (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 37-45°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 37°C to 45°C for 24-48 h for selection of efficient thermotolerant melanoidin decolorizing bacterium and yeast. Of the ten efficient bacterial isolates, B-25 strain was found the most potent melanoidin decolorizer as evident by maximum decolorization activity measured by spectrophotometer. Similarly, out of the eight efficient yeast isolates, RG-9 strain was found more effective for decolorization. The capability to grow at 37-45°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* and *Candida* sp. by IMTECH, Chandigarh (India).

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

Parameters	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

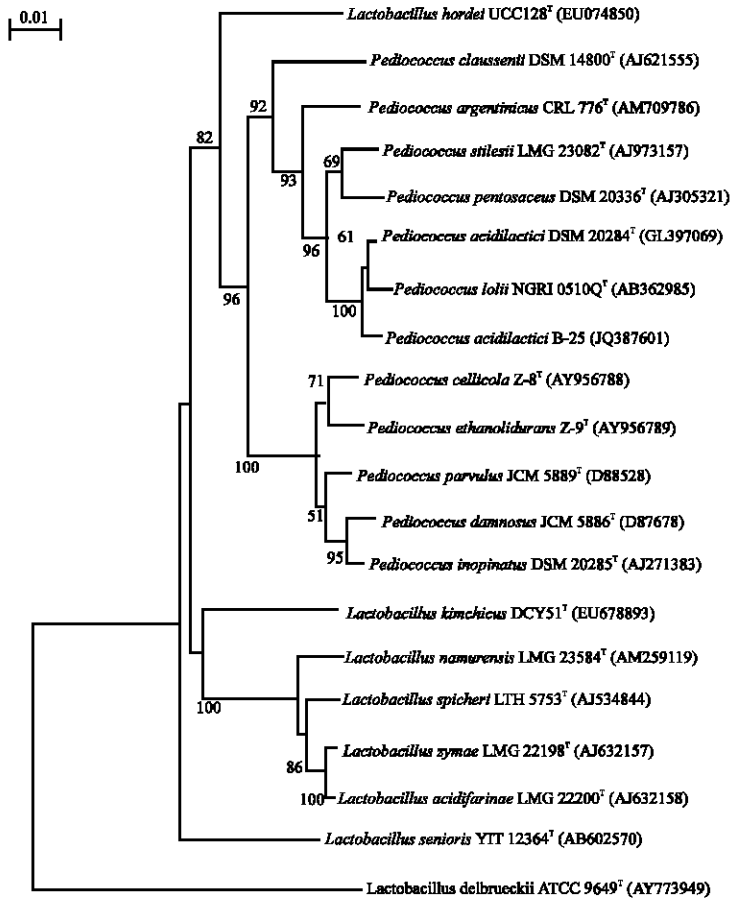

Fig. 2: Phylogenetic tree showing relation between Strain, B-25 and other *Pediococcus* strains

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 16 S rDNA sequence was submitted to Genebank [JQ: 387601]. The strain B-25 was in the same cluster of phylogenetic tree (Fig. 2) with different strains of *Pediococcus acidilactici*. However, the 16 S 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 2).

Development of consortium for melanoidin decolorization in column system: This experiment was conducted to verify whether molasses wastewater can be decolorized more effectively by mixed culture than the single bacterial or yeast. The effective strain of bacteria and yeast was used for consortium development. Different experiments were carried out for selection of efficient bacteria and yeast individually (data not shown). The best strain of bacteria and yeast was used for consortium development. In case of bacterial strain *Pediococcus acidilactici* B-25 and in case of yeast strain *Candida tropicalis* RG-9 was showed maximum decolorization. So, consortium of B-25 and RG-9 was developed and immobilized in continuous column system for better melanoidin decolorization.

Table 2: Morphological, physiological and biochemical characteristics of the selected RG-9 isolate

Morphological tests		Assimilation tests (Hydrolysis)	
Characteristics	Results	Characteristics	Results
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		D-Arabinose	_
Growth at 37-45°C	+	Inositol	_
Biochemical test		Erythritol	_
Gas prodnction from		Galactitol	_
Glucose	+	Melibiose	_
Maltose	+	Raffinose	_
Galactose	+	Lactose	_
Trehalose	+	L-Rhamnose	_
Sucrose	+	Capsules	_
Lactose	+	**Growth appearance on cornmeal**	
Assimilation tests		**and tween 80 agar**	
(Hydrolysis)		Branched	Present
Glucose	+	pseudohyphae	
D-Glucitol	+	Terminal vesicles	Absent
L- Arabinose	+	Ovoid blastoconidia	Present

+: Positive, -: Negative

Melanoidin decolorization by immobilized cells of the consortium in column system

Effect of different support materials on melanoidin decolorization: This experiment was conducted to optimized different support material for immobilization of consortium cells (2%, w/v) for better melanoidin decolorization. Maximum melanoidin decolorization (85%) was reported in sodium alginate (2%) when compared to control (free cells) followed by agar (Fig. 3). Agrose and polyacrylamide showed very less decolorization when compared to control as well as immobilized cells of the consortium.

Effect of different concentration of sodium alginate on melanoidin decolorization: In order to find out the optimum alginate concentration for entrapping consortium cells for better decolorization, different concentrations of sodium alginate were further evaluated. Maximum melanoidin decolorization (87%) was obtained by consortium at (2%) sodium alginate bead (Fig. 4). Therefore, immobilized cell of consortium at 2.0% showed better results as compared to free cells of consortium for melanoidin decolorization. Alginate at 2% was found to be the optimum concentration for formulation of globular and stable beads with better melanoidin decolorization and was used in all other experiments.

Effect of different concentration of calcium chloride on melanoidin decolorization: Different concentration of calcium chloride solution was also affected sodium alginate bead preservation and its effect on immobilization of cells on melanoidin decolorization. So, optimization

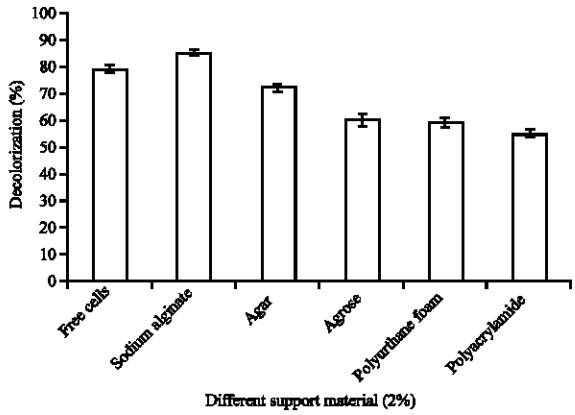

Fig. 3: Effect of different support material on melanoidin decolorization, the continuous column system was packed with different support material (having 50×10^6 CFU mL^{-1} microbial population) at 45°C temperature for 24 h. Error bars presented are mean values±standard deviation of triplicates of three independent experiments

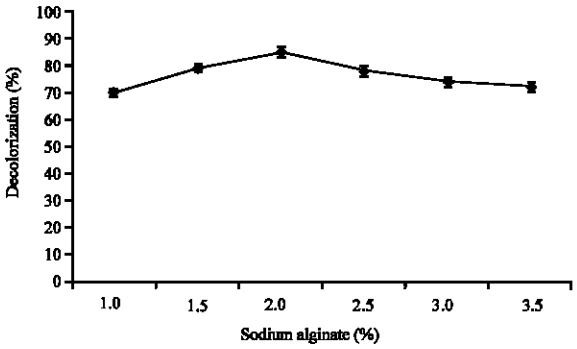

Fig. 4: Effect of different concentration of sodium alginate on melanoidin decolorization, the continuous column system was packed with different concentration of sodium alginate beads (having 50×10^6 CFU mL^{-1} microbial population) at 45°C temperature for 24 h. Error bars presented are mean values±standard deviation of triplicates of three independent experiments

of different concentration of calcium chloride was also carried out for better melanoidin decolorization in immobilized system. Results indicated that maximum decolorization (88%) was reported at 2.0% calcium chloride solution (Fig. 5). The immobilized cells prepared in 2% (w/v) $CaCl_2$ solution was found to be the best as it resulted in the highest decolorization. The percentage of decolorization did not change significantly within increasing of $CaCl_2$ concentration.

Effect of different curing time on melanoidin decolorization: To find the appropriate condition of calcium alginate beads were prepared with 2% alginate and 2% $CaCl_2$ solution with varying curing times (control, 10, 16 and 26 h). Among different curing time, higher melanoidin decolorization (89%) was reported in 16 h incubation at 4°C (Fig. 6). Increase of curing time resulted in a hared type of beads with less enzyme production. These results indicate that a curing time of 16 h is optimal for the formulation of stable calcium alginate beads and for better melanoidin decolorization.

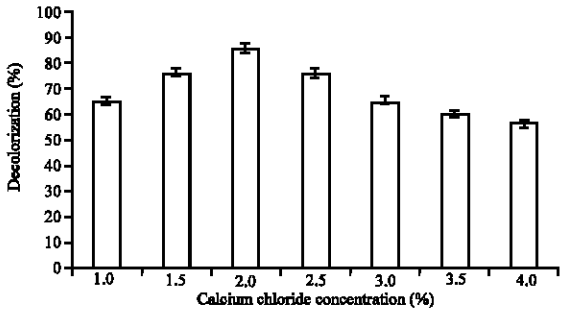

Fig. 5: Effect of different concentration of calcium chloride on melanoidin decolorization, Immobilized sodium alginate beads were prepared in different concentration of calcium chloride solution. Error bars presented are mean values±standard deviation of triplicates of three independent experiments

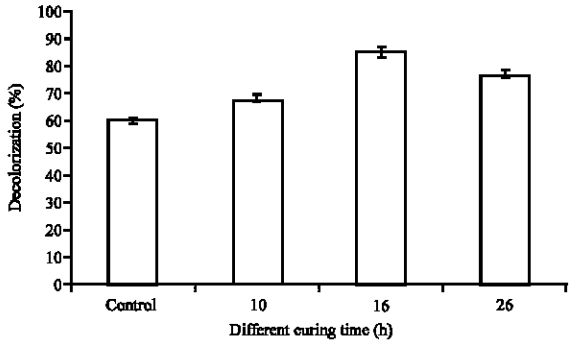

Fig. 6: Effect of different curing time on melanoidin decolorization, Sodium alginate beads were prepared in 2% calcium chloride solution and incubated at different curing time for bead stability. Error bars presented are mean values±standard deviation of triplicates of three independent experiments

Effect of different bead size on melanoidin decolorization: The sizes of sodium alginate beads in the column have also standardized for melanoidin decolorization. It is clear from the results that maximum melanoidin decolorization (90%) was found in 2 mm beads while smaller and larger than the 2 mm bead reduced the melanoidin decolorization significantly (Fig. 7). Hence, 2 mm size of the beads was used for column packaging for further optimization of the quantity of the beads for better decolorization. From Fig. 7 it observed that the smallest size of beads exhibited better decolorization when compared to large size beads.

Effect of different weight of beads on melanoidin decolorization: The next parameter in immobilization is different weight of the beads used in the column packaging for melanoidin decolorization. By varying the weight of beads from 5, 10, 15 and 20 g beads in the column, the influence of the initial beads number was tested. It was observed that the maximum decolorization (90%) level was obtained up to 5 g beads (Fig. 8). Decolorization yield decreased at high initial cell loading (5 g beads).

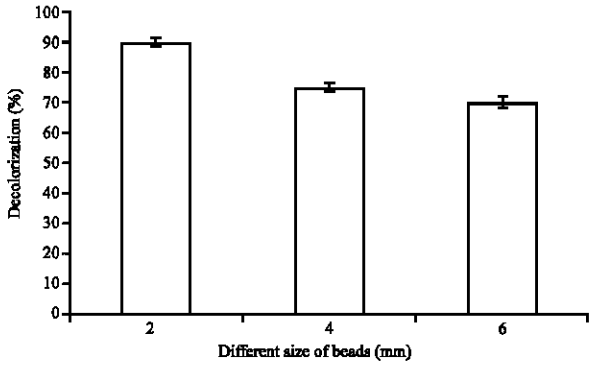

Fig. 7: Effect of different size of bead on melanoidin decolourization, different size of sodium alginate beads was prepared in 2% calcium chloride solution for immobilization. Error bars presented are mean values±standard deviation of triplicates of three independent experiments

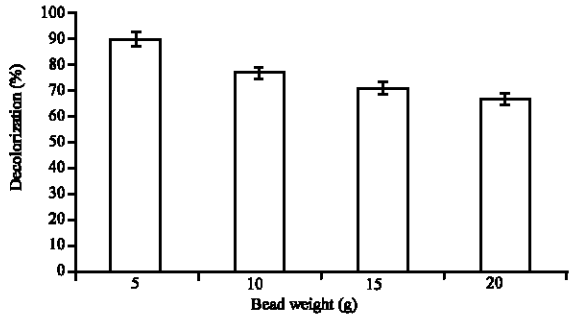

Fig. 8: Effect of different bead weight on melanoidin decolorization, Different weight of sodium alginate beads was used for packing column system for immobilization to achieve higher decolorization. Error bars presented are mean values±standard deviation of triplicates of three independent experiments

Effect of reusability cycle for melanoidin decolorization by immobilized cells of consortium: The number of cycles was calculated on the basis of capacity of the column that is, the release of 2.0 liters effluent from the column was considered as one cycle. It is clear from the result that in 18 cycles, no change in melanoidin decolorization was reported. Reduction in melanoidin decolorization was observed after 18 cycles (Fig. 9). Immobilized cells on alginate beads were reused in 18 successive reaction cycles, in which the medium of column released by the elution port having 2 L capacity, called one cycle (each cycle 24 h) without any loss of biocatalytic activity.

DISCUSSION

At present immobilized cells have been used in a variety of applications such as biotransformation, biosensors, production of ethanol, degradation of phenol etc. (Karandikar *et al.*, 2006; Prabakaran and Hoti, 2008; Abd-El-Haleem *et al.*, 2003; Jena *et al.*, 2005; Ahmed, 2008). Cell immobilization has some advantages when compared with free cell culture. The reaction speed can be accelerated, it is less susceptible to the effect of inhibitory compounds a nutrient depletion (Marques *et al.*, 2006), protect the cells against damage and reduced

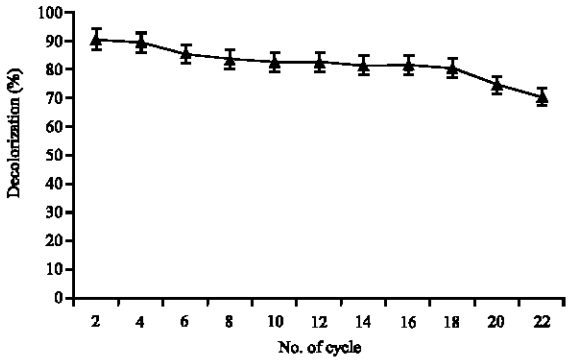

Fig. 9: Effect of reusability of beads in different cycle on melanoidin decolorization, optimized weight (g) of sodium alginate beads was packed in column system for their reusability in different cycle for immobilization to attain higher decolorization. Error bars presented are mean values±standard deviation of triplicates of three independent experiments

susceptibility to contamination (Prabakaran and Hoti, 2008). Cell immobilization increase productivity, stability, ease of separation and repeated use etc. also (Elibol and Moreira, 2003; Karandikar *et al.*, 2006). Thus, the present aim to optimize different parameters for melanoidin decolorizing by immobilized consortium cells of bacterium and yeast by continuous immobilized system. Melanoidin decolorization of molasses wastewater was recorded at different time intervals in different designs of the column along with immobilization unit keeping different support material in different quantity. The important feature of immobilization of cells in column technique is easy to cut down the temperature increase and pH of the medium, in which immobilization was carried out. Therefore, immobilization experiment was conducted a t room temperature with pH 6.0 for continuous melanoidin decolorization. The immobilization parameters were optimized in a continuous column system in order to achieve continuous melanoidin decolorization.

Immobilized cells of consortium on different support material (2%) were tested for melanoidin decolorization. Maximum melanoidin decolorization (85%) was reported in sodium alginate (2%) when compared to control (without immobilized) followed by agar (Fig. 3). Among different immobilization methods, gel entrapment is the most common (Nigam, 2000). Entrapment of cells in alginate is one of the simplest, cheapest and non-toxic that most frequently used method of immobilization (Adinarayana *et al.*, 2004). It also provides mild and physiological conditions for cell entrapment (Navratil *et al.*, 2001). The major limitations which may need to be addressed while using such cells are dispersion of cells, flow of nutrients away from cells, diffusion of substrate and products through the cell wall and unwanted side reactions due to the presence of other enzymes (Karandikar *et al.*, 2006; Ahmed, 2008).

In order to find out the optimum alginate concentration for consortium cells immobilization, different concentrations of sodium alginate were further evaluated for decolorization efficiency, the consortium showed maximum melanoidin decolorization (87% at 2%) sodium alginate (Fig. 4). Therefore, immobilized cell of consortium at 2.0% showed better results as compared to free cells of consortium for melanoidin decolorization. From Fig. 4 it observed that alginate concentration plays a major role in the production of enzyme responsible for decolorization by immobilized cells. It was observed that the enzyme titer was reduced with increased alginate concentration which may be due to reduced porosity of the beads restricting the nutrient supply and oxygen diffusion. Similarly, Elibol and Moreira (2003) also reported that 2% sodium alginate showed best result as best matrix

for immobilization purpose. Low alginate concentration resulted in leakage of biomass out the beads which might be due to increase in pore size of the beads (Beshay, 2003; Ahmed, 2008). Alginate at 2% was found to be the optimum concentration for formulation of globular and stable beads with better enzyme production for melanoidin decolorization and was used in all other experiments.

Different concentration of calcium chloride solution was also optimized for immobilization of cells and its effect on melanoidin decolorization. Maximum decolorization (88%) was reported at 2.0% calcium chloride solution (Fig. 5). The concentration of $CaCl_2$ is important for the stability and pore size of the bead (Elibol and Moreira, 2003). Immobilized beads were prepared with the same amount of cells in 2% sodium alginate solution using different $CaCl_2$ concentration. The immobilized cells prepared in 2% (w/v) $CaCl_2$ solution was found to be the best as it resulted in the highest enzymatic activity for better decolorization. These results are similar to that reported by Elibol and Moreira (2003) on the production of protease by immobilized *Teredinobacter turnirae* cells. The enzyme level for decolorization did not change significantly within increasing of $CaCl_2$ concentration.

To find the appropriate condition of calcium alginate beads were prepared with 2% alginate and 2% $CaCl_2$ solution with varying curing times (control, 10, 16 and 26 h). Among different curing time, higher melanoidin decolorization (89%) was reported in 16 h incubation at 4°C (Fig. 6). Increase of curing time resulted in a hared type of beads with less enzyme production (Adinarayana *et al*., 2004; Ahmed, 2008). These results indicate that a curing time of 16 h is optimal for the formulation of stable calcium alginate beads and better enzyme production for melanoidin decolorization.

The sizes of sodium alginate beads in the column have also standardized for melanoidin decolorization. It is clear from the results that maximum melanoidin decolorization (90%) was found in 2 mm beads while smaller and larger than the 2 mm bead reduced the melanoidin decolorization significantly (Fig. 7). Hence, 2 mm size of the beads was used for column packaging for further optimization of the quantity of the beads for better decolorization. Mass transfer is an important consideration for immobilized cell growth. Although the diffusion of small substrate in alginate is the same as in water, the diffusion of larger molecules may be restricted (Li *et al*., 1996; Ahmed, 2008). Since the cell primarily grow near the bead surface, cell growth and production behavior may be influenced by the square of the bead diameter. For this reason, the production behavior of consortium cells immobilized in 2, 4, 6 mm beads was compared. From Fig. 7 it observed that the smallest size of beads exhibited better decolorization when compared to large size beads. This might be due to increased surface area of the beads which enhances the mass transfer.

The next parameter in immobilization is different weight of the beads used in the column packaging for melanoidin decolorization. By varying the weight of beads from 5, 10, 15 and 20 g beads in the column, the influence of the initial beads number was tested. It was observed that the maximum decolorization (90%) level was obtained up to 5 g beads (Fig. 8). Decolorization yield decreased at high initial cell loading (5 g beads). This could be attributed to the fact that, when the weight of beads increases, the nutrient/bead ratio decreases which may become limiting (Beshay, 2003; Ahmed, 2008). Therefore, this weight of beads was used for the rest of the study.

The number of cycles was calculated on the basis of capacity of the column that is, the release of 2.0 liters effluent from the column was considered as one cycle. The immobilization capability of consortium was already optimized on the sodium alginate. It is clear from the result that in 18 cycles, no change in melanoidin decolorization was reported. Reduction in melanoidin decolorization was observed after 18 cycles (Fig. 9). The most important parameter for the purpose of immobilization is reusability of immobilized cell for melanoidin decolorization. Batch culture is

a common mode for commercial fermentation. In addition to fermentation time, however, the production cycle also includes turn around time (needed for sterilization, inoculation, turnaround etc.) which leads to a reduction in overall productivity and add to production costs (Elibol and Moreira, 2003; Ahmed, 2008). The semi-continuous fermentation was terminated to investigate the stability of the biocatalyst and its ability to produce enzyme for decolorization under repeated batch cultivation condition using optimized alginate beads (Beshay, 2003).

Immobilized cells on alginate beads were reused in 18 successive reaction cycles, in which the medium of column released by the elution port having 2 L capacity, called one cycle (each cycle 24 h) without any loss of biocatalytic activity. This result is higher than that obtained by Karandikar *et al.* (2006) on using immobilized cells five cycles for invertase production. From this result it clear that free cell does not exist to much time in the medium, because after a time microorganism reached its death phase. In the case of bind cells they exist for a long time because these cell in its stationary phase and they can not multiply more as free cells. The main aim of immobilization for decolorization was done at a scale for a long time without preparing the inoculum properly. The cost effective and fast melanoidin decolorization can only be achieved by continuous fermentation process which requires immobilization of suitable microbial consortium on a suitable matrix. It is clear from the findings that continuous decolorization by immobilized cells is better over the single culture for this microbial decolorization. For immobilization, the sodium alginate was used as solid matrix which could provide sufficient oxygen as well as microbial film forming capability into the fermentation medium simultaneously to achieve higher decolorization at constant rate. It is expected that in the large scale fermentation, column temperature increases 8-10°C due to high microbial activity, therefore, temperature tolerant microorganisms may survive. The microorganisms used for consortium development are thermotolerant, hence suitable for immobilization in continuous column system.

CONCLUSION

Immobilization of consortium cells in continuous column system is better than free culture. Among the immobilized cell bioreactors, no doubt that continuous column immobilization is a novel and efficient one which can be adopted for the treatment of industrial wastewater containing melanoidin compounds and other pollutants. A proper choice of immobilized culture, careful consideration of various design parameters for continuous column immobilization will make treatment process cost effective in the long run.

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