Journal of Applied and Natural Science 3 (1): 68-74 (2011)

JANS

Applications of submerged fermentation for biodegradation and decolourisation of melanoidins by an isolate *Alcaligenes denitrificans* SAG_5

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Abstract : In laboratory conditions a bacterium was isolated, which was identified as *Alcaligenes denitrificans* $SAG_{5.}$. The optimum decolourisation (72.6%) of melanoidin was achieved at pH 7.5 and temperature 37 °C within 4-6 days fermentation. The toxicity evaluation of distillery effluent with mung bean (*Vigna radiata*) revealed that the raw effluent is highly toxic as compared to treated effluent. This indicated that the effluent after bacterial treatment is eco-friendly.

Keywords: Fermentation, Melanoidins, Biodegradation, Decolourisation, Distillery effluent

INTRODUCTION

The increase in number of distilleries has resulted into substantial increase in industrial pollution in the global world. These distilleries are integrated with sugar mills and using molasses as a raw material for the production of ethanol. After processing, this raw material converted into waste and discharged into the water bodies. This waste water is known as effluent. Untreated (Raw) distillery effluent or spent wash is well known to cause pollution in the natural streams. It has also been shown that raw distillery effluent contains excess amounts of organic and inorganic loading and has high-acidic pH (Table 1). The disposal of wastes from industrial sources is becoming a serious problem throughout the world. In recent years, industrial effluents have been regarded as common source of pollution, because of lack of efficient treatment and improper mode of disposal of effluents generated by industries. Under these circumstances aquatic life suffers, resulting in loss of productivity natural water and deterioration of water quality to such an extent that the water becomes unusable (Chandra and Sirivastava, 2004).

Indian distilleries employ various forms of primary, secondary and tertiary treatments of wastewater. However, these treatments are highly energy intensive and hence quite expensive. Waste water treatment by conventional biological treatment leads to the large reduction in the organic load but colourants are scarcely degraded. This fact makes unfeasible the direct disposal of the effluent because coloured compounds are toxic and may reduce sunlight penetration in natural ecosystems and hence damaging the aquatic life. Colour can be removed by specific treatments such as coagulation-flocculation (Pandey *et al*., 2003), ozonation (Sirivastava *et al*., 2006) and adsorption on activated carbon (Chandra and Pandey, 2001). However, these processes have disadvantages due to high operation cost. These disadvantages emphasized the need for further research using biological methods. This method for removal of melanoidins before discharging into environment is necessary. The main objective of this study is to purify the wastewater by removing the colour and the contaminants by using bacteria.

Many researchers have tried to use biological processes for removing melanoidins from effluent. The ability to remove melanoidins from distillery effluent was studied in *Lactobacillus plantarum* (Tondee and Sirianuntapiboon, 2008); *Bacillus licheniformis*, *Bacillus* sp. and *Alcaligenes* sp. (Bharagava *et al*., 2009); *Klebsiella oxytoca, Serratia mercescens* and *Citrobacter* sp. (Jirianuntipon *et al*., 2008); *Pseudomonas aeruginosa* PAO1, *Stenotrophomonas maltophila*, and *Proteus mirabilis* (Mohana *et al*., 2007); *Bacillus thuringiensis* (MTCC 4714), *Bacillus brevis* (MTCC 4716) and *Bacillus* sp. (MTCC 6506) (Kumar and Chandra, 2006); *Pseudomonas putida, Aeromonas* sp. (Ghosh *et al*., 2002). Therefore, keeping the complexity of the distillery effluent in mind, we screened new bacteria that decolourise melanoidins wastewater. Hence, the efforts were directed towards the biodegradation and decolourisation of melanoidins present in the distillery effluent.

MATERIALS AND METHODS

Media used: King's B broth media (K_2HPO_4, 0.15%; $MgSO_4$, O.15%; Peptone, 2%; Glycerol, 1% v/v) and Melanoidin pigment broth (MPB) media (K_2HPO_4, 0.15%; $MgSO_4$, 0.15%; Peptone, 2%; Glycerol, 1% (v/v); Melanoidin, 1% (w/v) were used. Whenever the solid medium was required, the Agar-agar @ 2% (w/v) was added.

Table 1. Physico-chemical parameters of untreated (Raw) distillery effluents (An *et al*., 2002).

Parameters	Untreated distillery effluent
Colour	Dark brown
pH	4.5
Suspended solids	4578
Dissolved solids	16,866
Total solids	21,444
Oil	52
BOD	7752
COD	13,824
Total nitrogen	604.8
Chlorides	1300
Sulphates	120
Phenol	34
Phosphates	2.0
Cadmium	1.2
Copper	1.71
Lead	0.48

Except pH and colour, all values are in mg/L.

Microorganisms: All the microorganisms used in the present investigations were isolated from different sites of distillery effluent treatment plant. *Pseudomonas putida* (MTCC No. 2445) was used as a standard/ reference culture.

Distillery effluent: Distillery effluent was collected from effluent treatment plant. Main site was near by the oxidation ponds, where effluent was stored after anaerobic (primary) and aerobic (secondary) treatment before disposal.

Soil samples: Soil samples were aseptically collected from 6 sites of distillery located in Yamuna Nagar district of Haryana (India). Different sites of distillery plant were; effluent treatment plant, the site where the soil has been used since very long time for the disposal of effluent, new sites of disposal, nearby of running waste, dry samples and mud samples.

Melanoidin preparations: In melanoidins preparation, 0.05 Mol of glucose (sugar, 9.00 g) and 0.05 Mol of glycine (amino acid, 3.75 g) were dissolved in 20 ml of distilled water. The carbonyl compound–amino acid mixture was placed in an oven at 125 °C. The mixture was heated for 2 hr without covering. The solid was transferred to a mortar and carefully grounded to a fine powder (Adams *et al*., 2003).

Table 2. Effect of treated and raw effluent on germination of *Vigna radiata*.

Parameter (s)	Treated effluent	Raw effluent
% germination	83.2	33.3
Germination speed (Days)	7.40	3.33
Emergence index	8.3	2.0
Peak value	7.40	3.33
Vigor index	342	0.602
Shoot length (cm/seed)	3.713	0.072
Root length (cm/seed)	3.02	0.163
Wet root weight (mg/seed)	47.11	2.7
Wet shoot weight (mg/seed)	85.43	18.1
Dry root weight (mg/seed)	2.52	0.516
Dry shoot weight (mg/seed)	10.01	6.03
Number of leaves	1.40	0.166

All values are average of three replicates.

Isolation: Isolation of bacterial species was carried out from soil contaminated with effluent from distillery industry using enrichment culture technique. Each soil sample (10 g) was suspended in effluent amended broth media (90 ml) aseptically and incubated at 37 °C. After 8d, 50 ml from each flask was discarded and fresh 50 ml effluent amended broth was added so that desirable microorganisms could grow and again incubated for 8d. After 8d, an aliquot (100 µl) was spread on King's B agar plates and incubated at 37 °C.

Purification and characterization of isolates: Individual colonies of bacteria were picked up and subcultured to purify by streak plate method on King's B agar plates. Isolates were further characterized by biochemical tests and staining methods.

Screening of isolates having decolourisation activity: For inoculum preparation isolated microbes were cultured in King's Broth medium (pH - 7.0) for 24h at 37 °C. Decolourisation assay was performed for screening. In primary screening isolates were screened against various concentrations of melanoidins (0.2% - 2.0%, w/v). For primary screening the colour measurement was done by visual comparison. Selected isolates from primary screening were further used for secondary screening. In secondary screening decolourisation activity was expressed in terms of the percentage decolourisation (An *et al*., 2002).

Optimization: Various growth parameters required for the decolourisation activity by the isolate SAG_5 were optimized. These parameters included temperature (25 °C – 55 °C), pH (5.0 – 9.0), effect of various concentrations of distillery effluent (5% - 100%, v/v), supplementation of various carbon sources (maltose, sucrose, glucose, galactose, ribose, mannitol, fructose and xylose) at concentration of 2.0% (w/v), supplementation of various nitrogen sources (peptone, beef extract, ammonium nitrate, yeast extract, ammonium sulphate and sodium nitrate) at concentration of 0.5% (w/v), and supplementation of both selected carbon and nitrogen sources in combinations. The decolourisation ability of isolate SAG_5 was checked at optimum conditions.

Toxicity test: Toxicity test of distillery effluent was studied in mung bean (*Vigna radiata*). A laboratory experiment was designed to observe the toxicity effect of bacterial treated effluent on seed germination using Petri dish method (Sharma *et al*., 2002). The seeds were surface sterilized with 0.1% $HgCl_2$ for 2 min, followed with repeated washings by using sterilized distilled water. For each treatment three replicates and in each replicate 30 seeds were taken. Seeds were spreaded on sterilized

Table 3. Morphology and biochemical tests of the isolate SAG_5.

Properties	Observation/Reaction	Properties	Observation/Reaction
Colony morphology		H_2S production	-
Configuration	Round	Gas production	-
Margin	Entire	Casein hydrolysis	-
Elevation	Convex	Esculine hydrolysis	-
Surface	Smooth	Nitrate reduction	+
Pigment	Off white	Catalase test	+
Opacity	Translucent	Oxidase test	+
Gram's reaction	-ve	Urea hydrolysis	-
Cell shape	Rods,	Tween 20 hydrolysis	-
Size	0.5 - 1.5 µ	Tween 40 hydrolysis	-
Spore (s)	-	Tween 60 hydrolysis	-
Motility	+	Tween 80 hydrolysis	-
Growth on MacConkey	+, LF	Acid production from	
Indole test	-	Dextrose	+
Methyl Red test	-	Lactose	-
Citrate utilization	-	Sucrose	-

petri dishes lined with sterilized filter paper. The seeds were cultivated with equal volume (10 ml) of raw and treated distillery effluent. The Petri dishes were kept at room temperature. Germination measurements were made on 15th day from the day of sowing. Criterion for germination was visible protrusion of the seed coat.

RESULTS AND DISCUSSION

Isolation and screening: Sixty bacterial isolates were obtained and four efficient isolates were chosen after secondary screening. Different concentration (0.2- 2.0%) of melanoidins were tested to check the decolourisation efficiency of isolates. Isolate SAG_5 showed 58.8% decolourisation activity. 1.0 % concentration of melanoidins was selected for further studies (Fig. 1).

Effect of temperature: The effect of temperature on the decolourisation activity was examined over the range of 25 °C - 55 °C and the optimal temperature was found to be 37 °C (Fig. 2). Further increase of temperature upto 55 °C inhibited the growth of this isolate. Effluent decolourisation is affected by incubation temperature. Increase in temperature from 20 °C to 37 °C was accompanied with increase in decolourisation. Further increase in temperature to 40 °C adversely affected the decolourisation activity (Mohana *et al.*, 2007). Earlier, reported strain no. BP103 showed higher decolourisation yield of 76.4 ± 3.2% when cultivated at 30 °C (Sirianuntapiboon *et al.*, 2004a).

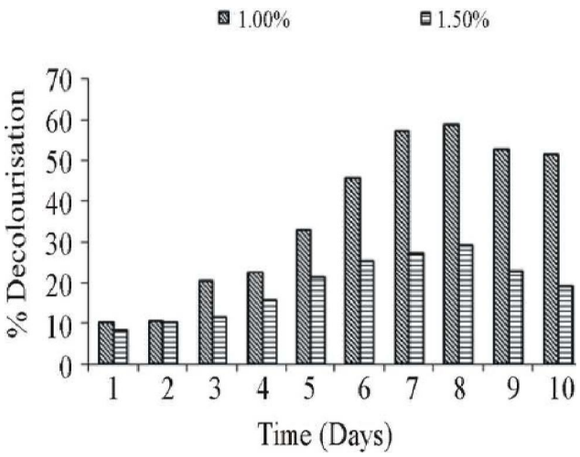

Fig. 1. *Decolourisation by isolate SAG_5 at 1.0% and 1.5% concentration of melanoidin in 8 d.*

Effect of medium pH: The effect of medium pH on decolourisation was examined over the range of (5.0 – 9.0) and the highest decolourisation activity was observed at pH 7.5 (Fig. 3) while no growth has been shown at pH 9.0. Maximum decolourisation yield was noted at pH range of 4.0 - 4.5 (Ohmomo *et al.*, 1988). For *Citeromyces* sp. WR-43-6 maximum activity was at pH 6.0 (Sirianuntapiboon, 2004b). However, acetogenic bacteria showed highest decolourisation activity at pH range of 5.0 - 7.0 (Sirianuntapiboon *et al.*, 2004a).

Effect of various concentrations of distillery effluent: Various concentrations (5.0% - 100%, v/v) of distillery effluent were studied. The maximum decolourisation showed by the isolate SAG_5 was 46.0% at 5.0% (v/v) concentration (Fig. 4). It was noticed that decolourisation increased with decrease in the concentration of distillery effluent.

Effect of carbon sources: The presence of a readily available carbon sources was necessary for growth of isolated culture and for melanoidin decolourisation. The decolourisation activity of SAG_5 on the medium containing various types of sugars as a carbon source is shown in Fig. 5. The isolate SAG_5 showed the high level of decolourisation yields (63.2%, 61.1%) within 5 days incubation when glucose or sucrose was used as a carbon source. Glucose (3.0%) and fructose (2.0%) showed highest decolourisation of 55.0 ± 1.2 % and 54.8 ± 0.71 % within 5 days cultivation (Sirianuntapiboon *et al.*, 2004a). Increase in concentration of glucose was essential for

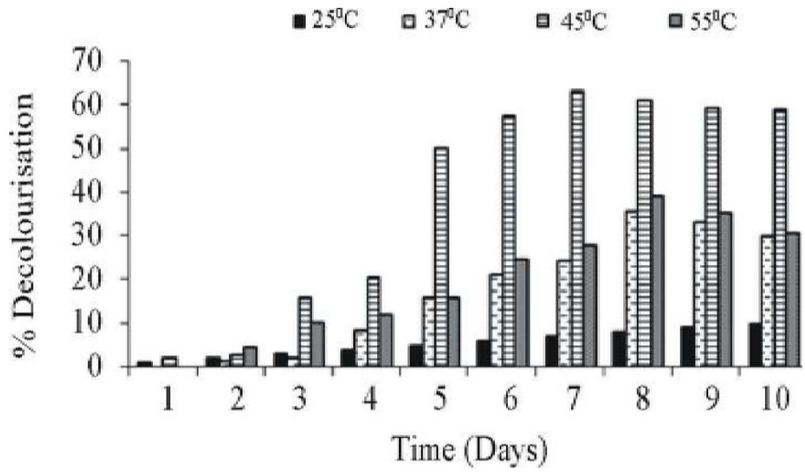

Fig. 2. *Effect of temperature on the decolourisation ability of isolate SAG_5.*

the growth and decolourisation activity of a yeast strain (*Issatchenkia orientalis*) (Tondee and Sirianuntapiboon, 2006). Ghosh *et al*. (2002) reported the use of 1% glucose obligatory for growth and decolourisation activity of *Pseudomonas putida.* Sirianuntapiboon *et al*. (2004b) reported that *Citeromyces* sp. WR-43-6 showed maximum decolourisation with glucose at concentration of 2.0% within 8 days of incubation. According to Mohana *et al*. (2007) the consortium DMC decolourised (67%) the anaerobically treated distillery spent wash in presence of basal salts and glucose (0.5%) and hence supplementation of glucose appeared to be necessary for decolourisation. Fructose, sucrose and galactose were found to be fairly good substrates allowing 60% – 67% decolourisation and 40% - 48% COD reduction. Isolated strains (BP103 and 13A) of acid forming bacteria showed maximum decolourisation with maltose (87.3%) and fructose (82.5%) within 5 days of incubation (Sirianuntapiboon, 1999).

Effect of nitrogen sources: The results of the effect of various nitrogen sources (0.5%, w/v) on melanoidin biodegradation and decolourisation showed that supplementation of nitrogen sources positively affected the bacterial decolourisation of effluent. The highest decolourisation yield (59.2%) within 5 days was observed using beef extract (Fig. 6). Sirianuntapiboon *et al*. (2004a) reported yeast extract and peptone (0.2%) as a nitrogen source that showed highest decolourisation of 55.8 ± 0.33% and 54.0 ± 0.81% within 5 days cultivation. According to Tondee and Sirianuntapiboon (2006) *Lactobacillus plantarum* No. PV71 - 1861 showed highest growth and melanoidin decolourisation when yeast extract was used as nitrogen source. Sirianuntapiboon *et al*. (2004b) examined that the inorganic nitrogen source was more effective than organic nitrogen source and the maximum decolourisation activity was 35.9% with $NaNO_3$ (0.1%) within 8 days of incubation. Sirianuntapiboon (1999) isolated two strains that showed highest decolourisation of 82.0% and 80.5% with yeast extract within 5 days. Mohana *et al*. (2007) found inhibitory effect of both the organic and inorganic nitrogen sources on melanoidin decolourisation by bacterial consortium.

Effect of supplementation of both selected carbon and nitrogen sources: In another experiment supplementation of both selected carbon (2.0%, w/v) and nitrogen (0.5%,

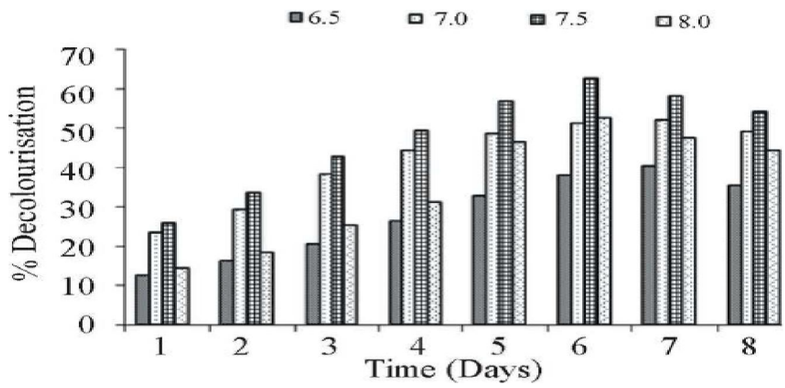

Fig. 3. *Effect of medium pH on the decolourisation ability of isolate SAG_5.*

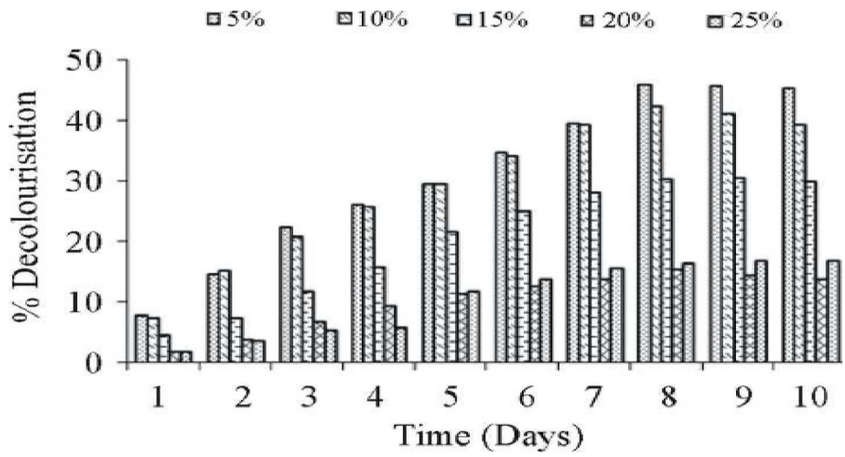

Fig. 4. *Effect of different concentrations of distillery effluent.*

w/v) source was used in the MPB. Four experimental setup included sucrose (+ beef extract or ammonium nitrate or yeast extract or peptone), glucose (+ beef extract or ammonium nitrate or yeast extract or peptone), maltose (+ beef extract or ammonium nitrate or yeast extract or peptone) and galactose (+ beef extract or ammonium nitrate or yeast extract or peptone) was performed. The isolate SAG_5 gave the maximum decolourisation yield (72.6%) within 5 days of cultivation with the supplementation of combination of glucose (2.0%) and beef extract (0.5%) (Fig.7). Sirianuntapiboon (1999) reported that the strain No. 13A and strain No. BP103 gave the maximum decolourisation in the presence of fructose (2.0%) and yeast extract (0.5%).

Decolourisation at optimum conditions: The isolate SAG_5 showed the highest decolourisation yield (72.6%) when it was cultured at 37 °C for 5 days with the supplementation of glucose (2.0%) and beef extract (0.5%) Sirianuntapiboon *et al.* (2004a) reported yeast extract and peptone (0.2%) as a nitrogen source that showed highest decolourisation of 55.8 ± 0.33 % and 54.0 ± 0.81 % within 5 days cultivation. However, isolate SAG_5 showed maximum decolourisation (63.2%) with glucose (2.0%) and 59.2% decolourisation with beef extract (0.5%) within 5 days.

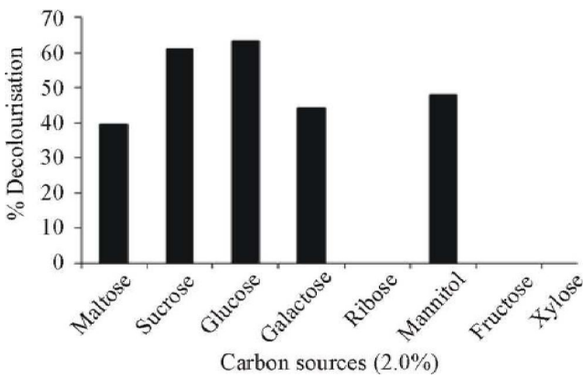

Fig. 5. *Effect of carbon sources on the decolourisation activity.*

Effluent toxicity test: Petridish having seeds with treated effluent showed initiation of germination on 3rd day whereas no germination was observed with raw effluent. Sharma *et al.* (2002) reported that industrial effluent have some toxic components that inhibit the germination of seeds. It was observed that the effluent before treatment was very toxic which inhibited the growth of seeds but after treatment the toxicity of effluent decreases (Plate 1). Various parameters were studied showed % germination (83.2%, 33.3%); germination speed (7.40, 3.33); emergence index (8.3, 2.0); and peak value (7.40, 3.33) with treated and raw effluent respectively. The plant growth measurements included shoot length, root length, wet shoot weight, wet root weight, dry shoot weight, dry root weight and vigor index were also noted (Table 2). The vigor index (342), shoot length (3.713 cm), root length (3.02 cm), wet root weight (47.11 mg), wet shoot weight (85.43 mg), dry root weight (2.52 mg), dry shoot weight (10.01 mg) and no. of leaves (1.40) were highest in treated effluent. According to Chandra and Sirivastava (2004) the untreated effluent could not support growth. The

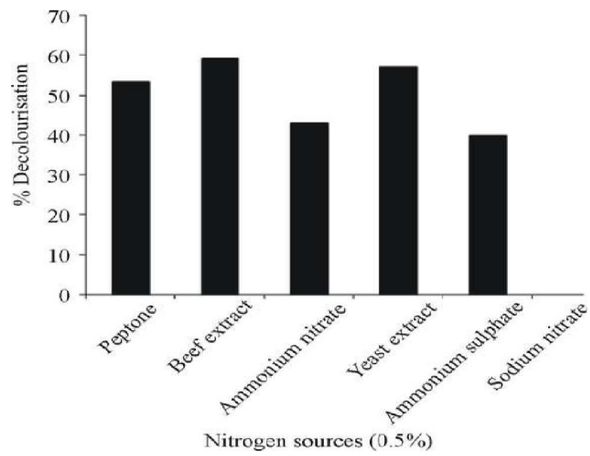

Fig. 6. *Effect of nitrogen sources on the decolourisation activity.*

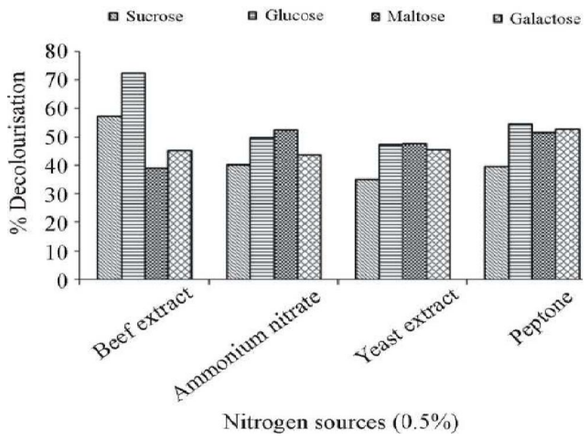

Fig. 7. *Combined effect of both selected carbon and nitrogen sources on the decolourisation activity.*

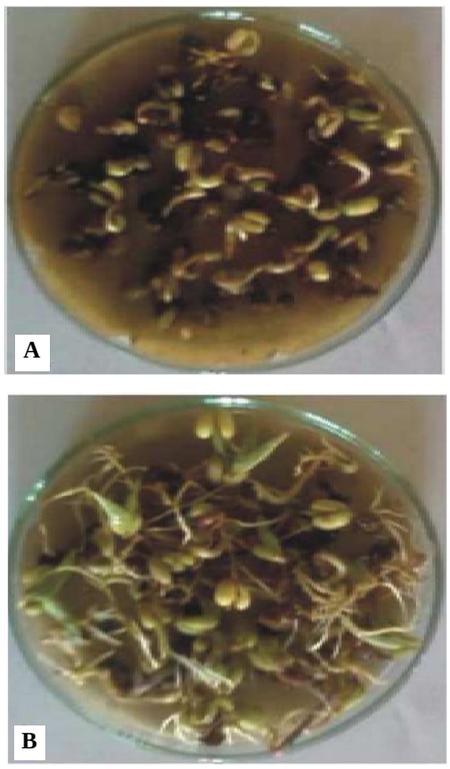

Plate 1. *Toxic effect of effluent on germination of seeds treated with raw effluent (A) and treated effluent (B).*

reason for this could be high concentrations of toxicants, because further dilution of the effluent supported the growth and showed less harmful effects on chlorophyll content, protein and biomass of the duckweed plant. The higher concentration of dissolved salts and cations are responsible for inhibition of the seed germination and related parameters (Suresha and Puttaiah, 2006). Reduction in seed germination and seedling growth treated with raw effluent may be due to higher amount of dissolved solids present in effluent. The dissolved solids may prevent the germination by contribution to salinity of the solute absorbed by the seeds before germination (Medhi, 2008). Therefore, we can conclude that after microbial decolourisation of anaerobically treated effluent reduced the toxic effect on which indicated that there is necessity for microbial degradation at secondary or tertiary stage prior to its disposal for environmental safety even after the existing anaerobic treatment system in industry.

Characterization and identification of isolate SAG_5 : On the basis of standard methods the isolate SAG_5 was found to be a strain of *Alcaligens denitrificans*. It was named as *Alcaligens denitrificans* SAG_5 and was given accession MTCC 9781 (Table 3).

ACKNOWLEDGEMENTS

The financial assistance received from University Grants Commission (UGC), Govt. of India is gratefully acknowledged.

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