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ORIGINAL ARTICLE

Decolourization of Distillery Spent Wash by *Bacillus coagulans* ABS012

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ABSTRACT
In concern to water pollution, cane molasses-based distilleries are considered as one of the most polluting industries. The use of molasses in distilleries produces a large amount of dark brown coloured spent wash with a high Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD). The distillery spent wash may cause several environmental hazards. The discharge of spent wash into adjacent water bodies and lands adversely affects the nearby aquatic and terrestrial habitats. Hence, a proper pre-treatment is required for its safe disposal in to the environment. In present study, decolourization of distillery spent wash was attempted by using the bacterial strain identified as Bacillus coagulans ABS012. Different environmental and nutritional parameters were optimized for maximizing decolourization. Dilution of spent wash up to 10 % and addition of nutrients had a favourable effect on decolourization of distillery spent wash. The maximum decolourization yield (44.30 %) was obtained at 35^{0}C using modified spent wash medium containing 1% (w/v), glucose; 0.3 % (w/v), peptone; 0.1% (w/v), yeast extract; 0.2% (w/v), K_2HPO_4; 0.1% (w/v), KH_2PO_4; 0.05% (w/v), $MgSO_4$; 0.01% (w/v), $NaNO_3$; 10 % (v/v), distillery spent wash; pH-6.0 within 5 days of incubation under static condition. The decolourization ability of the isolate was confirmed by HPLC analysis of treated sample. The seed germination test using Phaseolus mungo L. showed toxicity reduction in decolourized spent wash. The results of the present study will be helpful in development of cost-effective, environment friendly and publicly acceptable alternative to conventional methods for the decolourization of distillery spent wash.
KEYWORDS: *Bacillus coagulans*, decolourization, spent wash, static condition

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INTRODUCTION

Environmental pollution is one of the major problems faced by the modern world. Pollution control plays a significant role in sustainable industrial development. Hence, there is a need for proper effluent treatment to reduce pollutant loads. Sugar industry is considered as one of the most important agro-based industries in India having a large impact on the country's economy. Most of the Indian distilleries are associated with sugar industries and produce alcohol from molasses, a by-product of the sugar industry. The distilleries generate enormous amounts of effluent spent wash for every litre of alcohol produced [1]. The distillery spent wash is dark brown in colour having unpleasant smell, high Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and low pH [2, 3]. The distillery spent wash is a highly recalcitrant waste product because of its melanoidin content. The disposal of such a large amount of distillery effluent is one of the critical environmental issues. The spent wash is injurious to aquatic life because its coloured components affect penetration of sunlight which in turn reduce photosynthesis process and dissolved oxygen concentration in water bodies. Inappropriate land discharge of distillery effluent is also harmful because it inhibits seed germination, reduces alkalinity of soil and causes soil

manganese deficiency [3, 4, 5, 6]. Hence, pre-treatment is required in order to ensure safe disposal of distillery spent wash into the environment.

Several methods have been employed for decolourization of distillery spent wash, but with limited success so far. Common physicochemical methods for colour removal are expensive, require high reagent dosage, generate large amounts of sludge and hazardous by-products [1, 3, 7, 8]. They are also not eco-friendly. Melanoidins are toxic to many organisms due to their antioxidant properties. Therefore, the use of conventional biological methods for distillery effluent treatments is also not feasible [3, 7, 9]. In recent years, increased attention has been directed towards utilization of microbial activity for decolourization and bioremediation of distillery effluent.

Many authors have reported decolourization of distillery spent wash by using different microorganisms [1, 3, 6, 7, 10]. Many fungi have been investigated for their ability to decolourize distillery spent wash but decolourization of distillery spent wash by using bacterial culture have also been reported frequently. Bacteria have more potential for degradation of melanoidin compounds because they can adapt to a vast range of environmental conditions and possess biochemical versatility [3, 11, 12].

In the present investigation, a bacterial strain *Bacillus coagulans* ABS012 isolated from soil was used for decolourization of distillery spent and various process parameters were optimized for maximizing decolourization. To confirm melanoidin biodegradation, HPLC analysis of treated samples was carried out. The toxicity evaluation of distillery spent wash prior and after bacterial treatment was also performed by using seed germination test of *Phaseolus mungo* L.

MATERIAL AND METHODS

Distillery spent wash

The distillery spent wash was collected aseptically from the spent wash collection tank of the Distillation Unit of Vishwasrao Naik Co-operative Sugar Factory, Chikhali, Dist- Sangli, Maharashtra, India and stored at 4^0C in laboratory for further use [13].

Media Composition

The spent wash medium containing (1.0%, w/v), glucose; (0.5% w/v), peptone; (0.2% w/v), K_2HPO_4; (0.1% w/v), KH_2PO_4; (0.05% w/v), $MgSO_4.7H_2O$; and (10 % v/v); distillery spent wash was used throughout this study. The pH of the medium was adjusted to 6.0 using 0.1 N NaOH or 0.1 N HCl.

Preparation of bacterial culture

A bacterial strain *Bacillus coagulans* ABS012 capable of decolourizing distillery spent wash was used in this study. It was isolated from soil contaminated with distillery spent wash. The seed culture was prepared by inoculating a loopful of 24 hour grown culture on spent wash agar plate into 50 ml spent wash broth and incubated at 35^0 for 24 hours to achieve exponential phase.

Decolourization assay of the spent wash:

For decolourization assay, the bacterial isolate was inoculated in spent wash broth and incubated at 35^0C. After incubation, culture broth was centrifuged at 10,000 rpm for 10 min. The supernatant was taken and absorbance was measured at 475 nm using spectrophotometer. Uninoculated medium served as control. The decolourization yield was expressed as the decrease in the absorbance at 475 nm against initial absorbance at the same wavelength [1, 6, 7, 10].

Optimization of culture conditions for maximizing decolourization

Selection of physical parameters for decolourization

Effect of pH on decolourization

To find out the optimum pH for maximum decolourization, the pH of the medium was adjusted to 5.0 6.0, 7.0, 8.0 and 9.0. The medium was then inoculated with 0.5% (v/v) inoculums of the bacterial strain and incubated at 35^0C. The extent of decolourization was measured.

Effect of temperature on decolourization

In order to study the effect of temperature on the decolourization yields, the medium was inoculated with 0.5% (v/v) inoculums of the bacterial strain and incubated at different temperatures viz. 30, 35, 40 and 45°C. The extent of decolourization was measured.

Effect of incubation period on decolourization

To find out the optimum incubation period, the medium was inoculated with 0.5% (v/v) inoculums of the bacterial strain and incubated at 35^0C. The samples were drawn at a regular time interval of 24 hour for 8 days and the extent of decolourization was measured.

Selection of nutritional parameters for decolourization

Effect of carbon sources on decolourization

Different carbon sources viz. glucose, sucrose, fructose, maltose, lactose and starch at 1 % (w/v) were individually added in the medium and inoculated with 0.5% (v/v) of the bacterial strain separately with

their respective optimized pH and temperature and the decolourization yields were computed. Based on the decolourization potentials, the best carbon source was selected.

Effect of different concentration of the best carbon source

In order to optimize the level of the carbon source for maximum decolourization, the medium was supplemented with the best carbon source at 0.0, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 % (w/v) concentration and the decolourization yields were computed.

Effect of nitrogen sources on decolourization

Different organic and inorganic nitrogen sources viz. peptone, yeast extract, beef extract, ammonium chloride, ammonium sulphate and sodium nitrate were individually added into the medium at 0.5% (w/v) and inoculated with 0.5% (v/v) inoculums. Optimized concentration of carbon source was added to all. Based on the decolourization potentials, the best nitrogen source was selected.

Effect of different concentrations of the best nitrogen source

In order to optimize the level of nitrogen source for maximum decolourization, the medium was supplemented with the best nitrogen source at 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 % (w/v) concentration and the decolourization yields were computed.

Effect of different concentrations of spent wash

Different concentrations of spent wash viz. 10, 15, 20, 25 and 30 % (v/v) supplemented in medium were inoculated with 0.5% (v/v) of the bacterial strain and the decolourization yields were computed.

Selection of efficient medium for decolourization

An experiment was conducted to select an efficient medium for decolourization. Four types of media with different composition were used to assess decolourization potential of the isolate.

Medium 1: 1.0% (w/v), glucose; 0.3% (w/v), peptone; 0.2% (w/v), K_2HPO_4; 0.1% (w/v), KH_2PO_4; 0.05% (w/v), $MgSO_47H_2O$; 10 % (v/v), spent wash; pH 6.0.

Medium 2: 1.0% (w/v), glucose; 0.3% (w/v), peptone; 0.1% (w/v), yeast extract; 0.2% (w/v), K_2HPO_4; 0.1% (w/v), KH_2PO_4; 0.05% (w/v), $MgSO_47H_2O$; 0.01% (w/v), $NaNO_3$; 10 % (v/v) spent wash; pH 6.0.

Medium 3: 1.0% (w/v), glucose; 0.3% (w/v), peptone; 0.1% (w/v), KCl; 0.2% (w/v), K_2HPO_4; 0.1% (w/v), KH_2PO_4; 0.05% (w/v), $MgSO_47H_2O$; 0.001% (w/v), $FeSO_4$; 0.01% (w/v), $NaNO_3$; 10 % (v/v) spent wash; pH 6.0.

Medium 4: 1.0% (w/v), glucose; 0.3% (w/v), peptone; 0.1% (w/v), $NaNO_3$; 0.1% (w/v), KH_2PO_4; 0.05% (w/v), $MgSO_47H_2O$; 10 % (v/v) spent wash; pH 6.0.

HPLC analysis of treated spent wash

Decolourization of spent wash was monitored by HPLC (Thermo Finnigan Surveyor HPLC System). HPLC analysis was carried out at Hitech lab and Consultancy, Sangli, India.10 ml of samples were taken, and centrifuged, filtered through 0.45 µm membrane filter (Millipore). Filtered sample was analysed using a mobile phase consisting of acetonitrile and methanol (45:55) (HPLC grade) with 1ml glacial acid and 0.5 ml sodium acetate. The sample was eluted using C-18; reverse phase column of 5 µm SGE, 250 x 4.6mm SS. Resultant peak was analysed with UV detector 475 nm. The flow rate of the mobile phase was 1 ml/min [14-17].

Phytotoxicity assessment

The effect of untreated and bacterial treated distillery spent wash on seed germination of *Phaseolus mungo* L. was studied using the petri dish method as described previously [18-21]. Different concentrations of distillery spent wash viz. 2, 4, 6, 8 and 10 % (v/v) were used for the seed germination test. The seeds were surface sterilized with 0.1% mercury chloride ($HgCl_2$) solution for 2 min. Then, seeds were repeatedly washed with sterilized distilled water. Subsequently, ten seeds of *P. mungo* L. were placed in sterilized glass petri dishes lined with two Whatman no. 1 filter paper discs. These filter discs were then moistened with 10 ml of treated and untreated spent wash samples separately. The tap water was used for control. The petri dishes were kept at room temperature for a period of 3 consecutive days. Seed germination percentage was calculated by using methods described earlier [20-22].

RESULTS AND DISCUSSION

Bacterial culture

The bacterial strain *Bacillus coagulans* ABS012 used in this study was isolated from soil contaminated with distillery spent wash and has ability to decolourize distillery spent wash.

Optimization of culture conditions for maximizing decolourization

Selection of physical parameters for decolourization

The various parameters were optimized for maximizing decolourization. The effects of different pH, temperature and incubation period on decolourization were studied.

Effect of pH on decolourization

The influence of pH on decolourization of distillery spent wash was studied by varying the pH from 5.0 to 9.0.

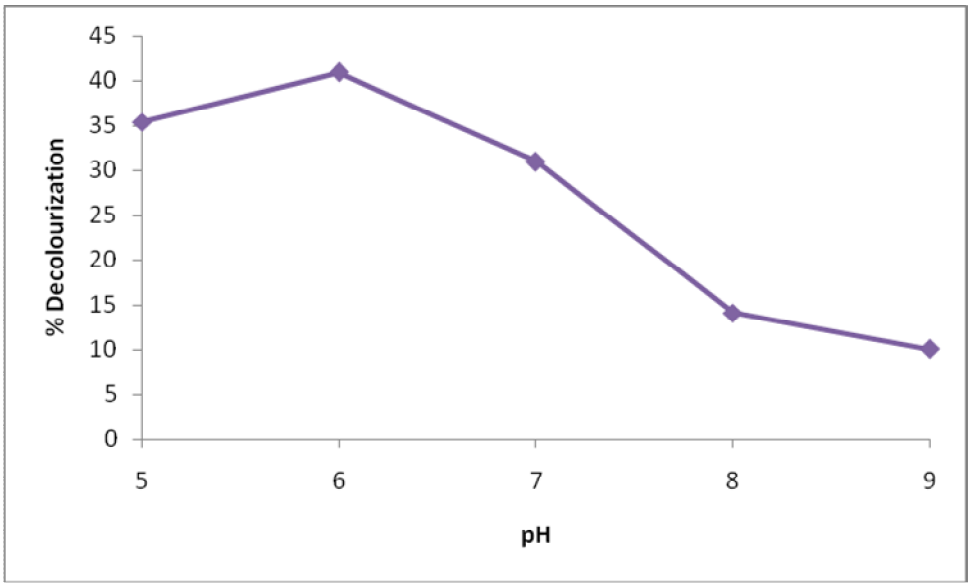

Fig 1: Effect of different pH on decolourization

The isolate showed better percentage decolourization with initial pH of 6. Any deviation from optimum pH reduced decolourization (Fig1). In previous study, it has been found that enzymes formed by microorganisms during the decolourization were effective only in acidic conditions [23]. It has also been reported that the polymerization of melanoidin and higher rate nutrient utilization are responsible for increase in colour at higher pH [24, 25]. The inhibition of enzyme production as well as activity may cause reduction in decolourization efficiency at above and below of optimum pH.

Effect of temperature on decolourization

The effect of temperature on decolourization of distillery spent wash was studied by varying the temperature from 30°C to 45°C.

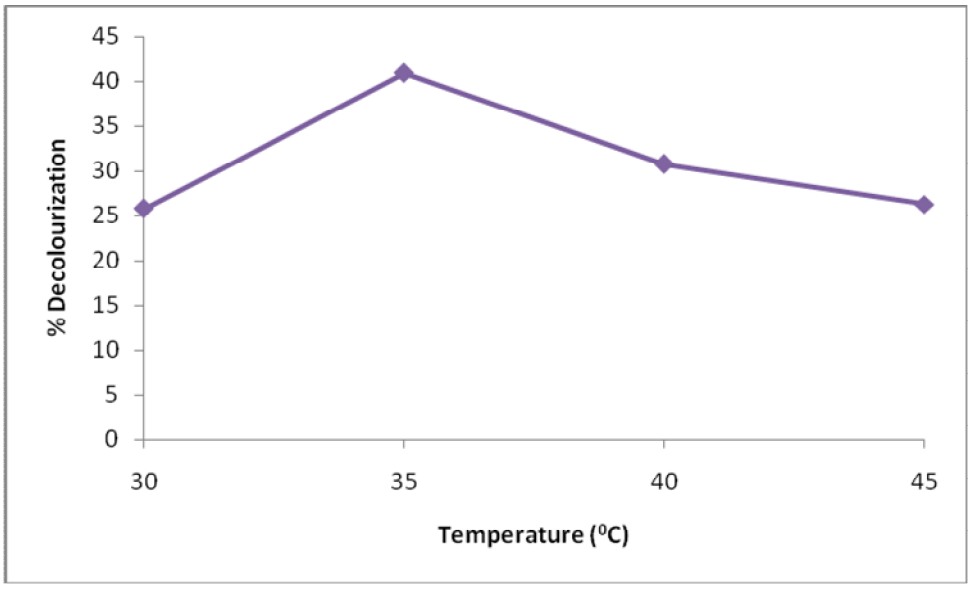

Fig 2: Effect of different temperatures on decolourization

Optimum temperature for decolourization was found to be 35^{0}C. Further increase in temperature caused reduction in decolourization ability of bacteria (Fig 2). Similar observations with respect to decolourisation pattern at variable temperature have also been reported in previous studies [1, 4, 26, 27]. The decolorizing activity is suppressed at high temperature due to loss in cell viability or deactivation of the enzymes responsible for decolorization [28].

Effect of incubation period on decolourization

Time course of distillery spent wash decolourization was studied. Maximum decolourization was achieved in a 5 days incubation period. Further increase in incubation period did not increase decolourization (Fig.3).

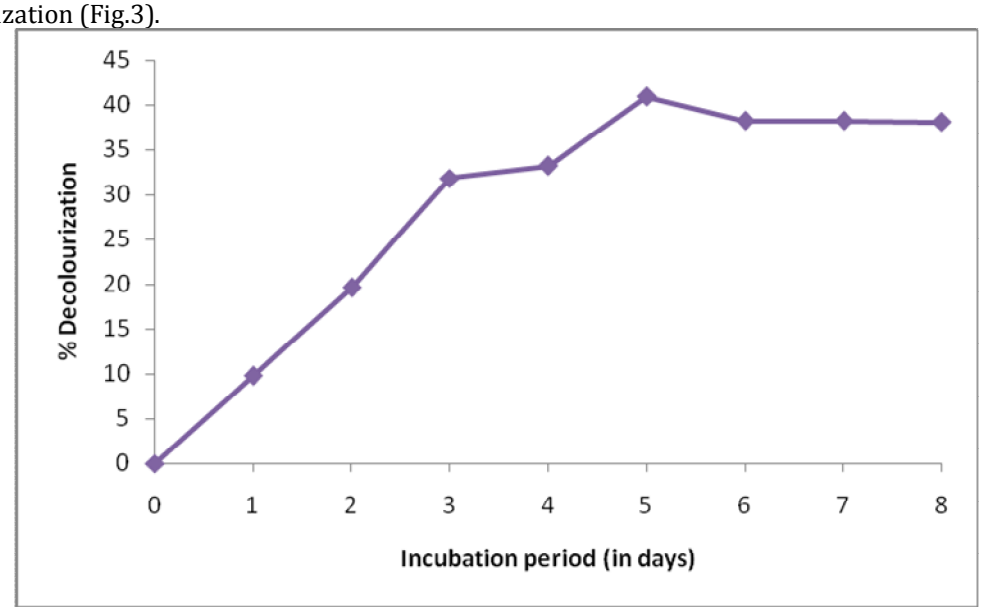

Fig 3: Effect of different incubation periods on decolourization

It was reported that *Issatchenkia orientalis* required 7 days of incubation to show maximum decolourization [29]. In another study, maximum decolourization was obtained using *Citeromyces* sp. WR-43-6 after 7 days of incubation [30]. It was observed that thermotolerant *Bacillus subtilis* required a very short period i. e. 24-48 hours of incubation to show maximum decolourization [8].

Selection of nutritional parameters for decolourization:

Effect of different carbon sources on decolourization

The effect of different carbon sources at 1 % (w/v) on decolourization of distillery spent wash was evaluated and results are represented in Fig. 4. The best carbon source for decolourization was found to be glucose. However, starch was found to be the least effective carbon source for decolourization of spent wash.

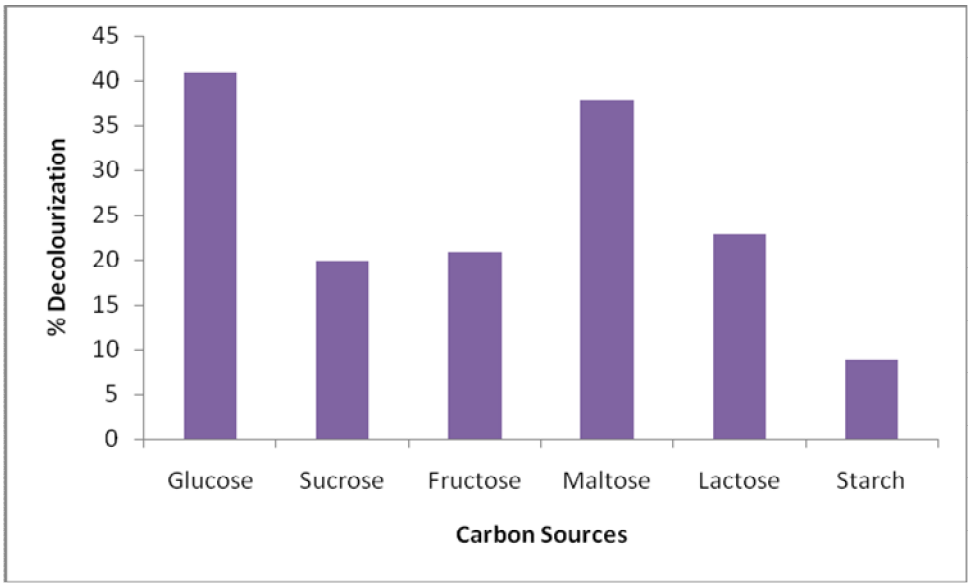

Fig 4: Effect of different carbon sources on decolourization

Spent wash contains a large amount of sugar but easily metabolizable carbon content of spent wash is almost negligible [31]. The presence of easily available carbon sources in medium increased decolourization efficiency. The organism utilizes easily available carbon sources present in the medium

during the initial growth phase and then it starts to degrade spent wash components for carbon source [31]. At optimal concentration, glucose may generate more redox mediators which might act as electron donors for the reduction and cleavage of conjugated C=C, C=O and C≡N bonds of melanoidins [4, 7, 32].

Effect of different concentration of the best carbon source on decolourization

It was observed that the decolourization efficiency increases with increase in glucose concentration from 0.1 to 1%. Maximum decolourization was observed in presence of 1 % glucose concentration. Further increase in glucose concentration did not increase decolourization (Fig.5). Increase in glucose concentration may result in formation of excess gluconic acid [4].

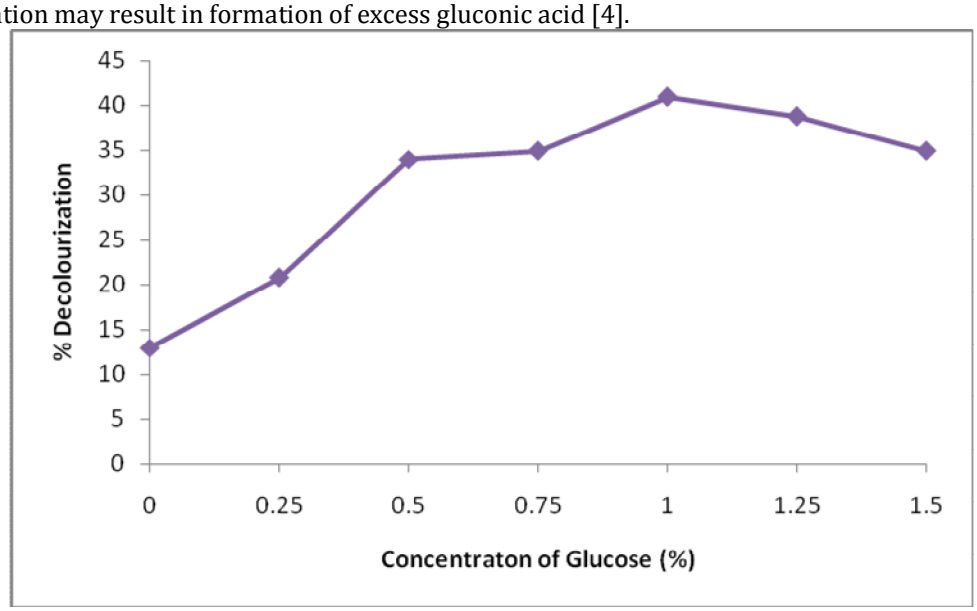

Fig 5: Effect of different concentrations of glucose on decolourization

Several workers have also reported similar observations during bacterial treatment of distillery spent wash [4, 12, 26].

Effect of nitrogen sources on decolourization

The effect of different organic and inorganic nitrogen sources at 0.5 % (w/v) on distillery spent wash decolourization was evaluated and results are represented in Fig. 6. The highest decolourization was obtained when peptone was used as a nitrogen source in the medium.

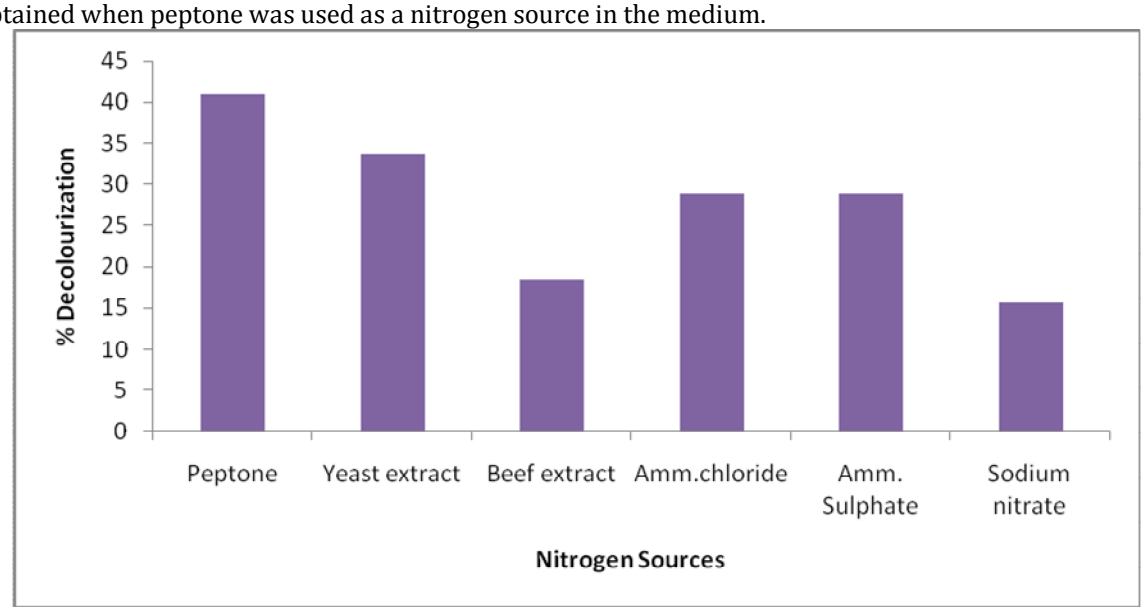

Fig 6: Effect of different nitrogen sources on decolourization

Beef extract and sodium nitrate were found to be least effective nitrogen sources for decolourization. The inhibitory effect of inorganic nitrogen sources for melanoidins decolourisation has been reported in previous studies [4, 6, 7]. However, *Issatchenkia orientalis* and *Citeromyces* sp. WR-43-6 showed maximum decolourization in the presence of 0.1% NH_4Cl and 0.1% sodium nitrate [29, 30]. It has also

been reported that degradation of lignin and lignin-like materials is catalysed by enzymatic systems during the secondary phase of the metabolic growth in the presence of peptone [34].

Effect of different concentrations of the best nitrogen source

The optimum concentration of peptone for decolourization was found to be 0.3 %. Further increase in peptone concentration inhibited decolourization process (Fig. 7).

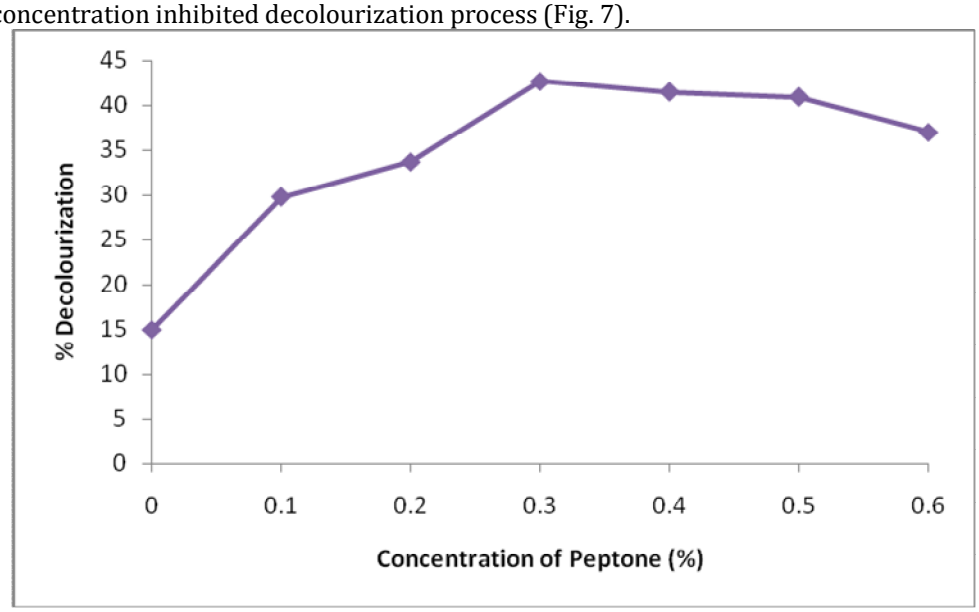

Fig 7: Effect of different concentrations of peptone on decolourization

This may be due to inhibition of bacterial growth by surplus addition of nitrogen source [4, 8].

Effect of different concentrations of spent wash

The decolourisation process was greatly influenced by concentration of distillery spent wash. Maximum decolourization was obtained at 10 % spent wash concentration. The decolourization efficiency decreased with further increase in spent wash concentration (Fig. 8). Similar observations have been reported in previous studies [34].

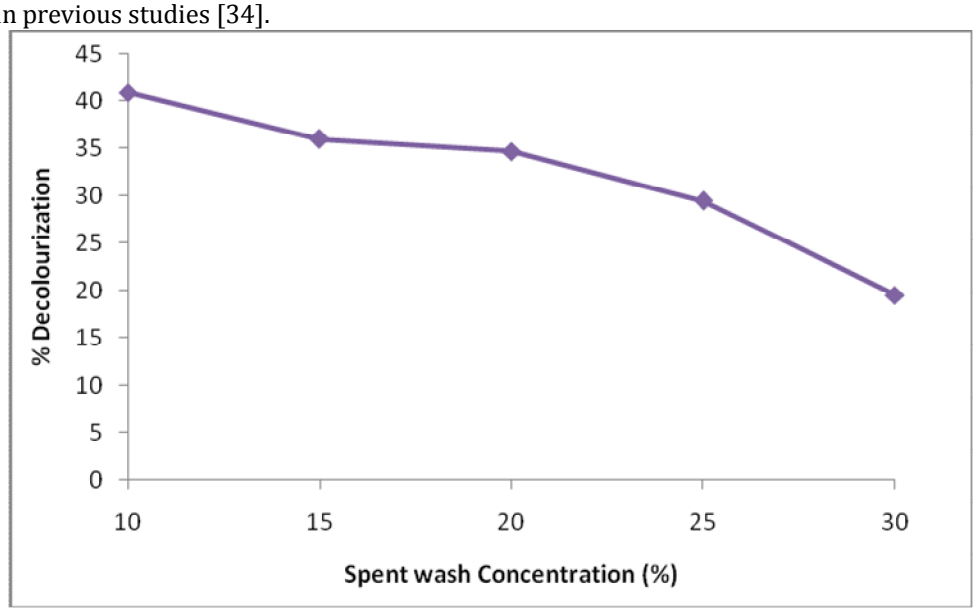

Fig 8: Effect of different spent wash concentrations on decolourization

Selection of efficient medium for decolourization

The Medium 2 containing (w/v) 1%, glucose; 0.3 %, peptone; 0.1%, yeast extract; 0.2%, K_2HPO_4; 0.1%, KH_2PO_4; 0.05%, $MgSO_4$; 0.01%, $NaNO_3$; 10 % distillery spent wash (v/v) was found to be most suitable for spent wash decolourization. 44.3 % decolourization was obtained by using Medium 2 (Fig. 9).

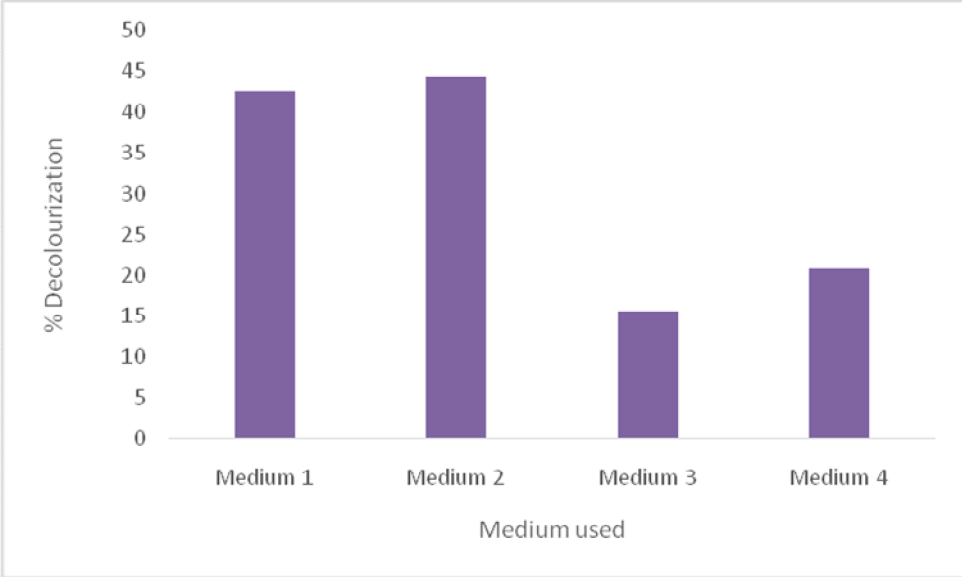

Fig 9: Selection of efficient medium for decolourization

HPLC analysis

The HPLC analysis of bacterial treated samples of spent wash after a 5 days incubation period has shown that there is reduction in peak areas compared to control (Fig. 10). It indicates that decrease in colour intensity might be due to the ability of bacterial isolate to decolorize and degrade the spent wash. The similar findings were reported by earlier workers [4, 17, 35, 36].

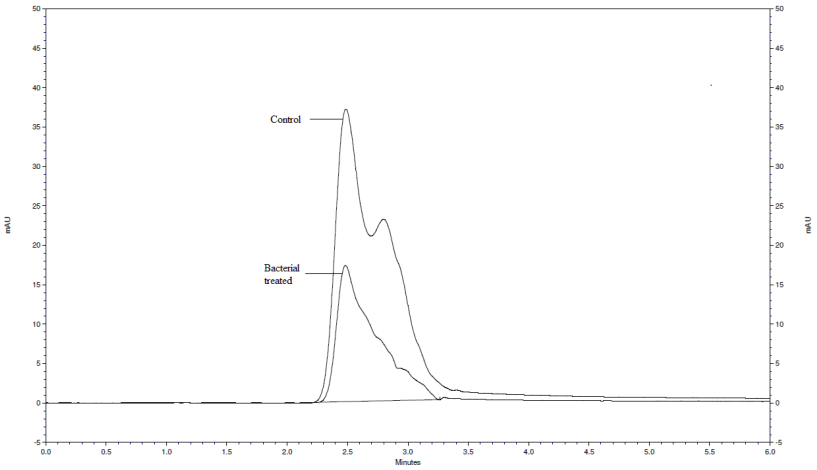

Fig 10: Comparative HPLC chromatogram of spent wash before and after bacterial decolourization

Phytotoxicity assessment

The results of the seed germination test revealed that up to 2% (v/v) concentration of untreated spent wash has no inhibitory effect on seed germination. As the concentration of spent wash increased, the percent seed germination decreased (Fig. 11).

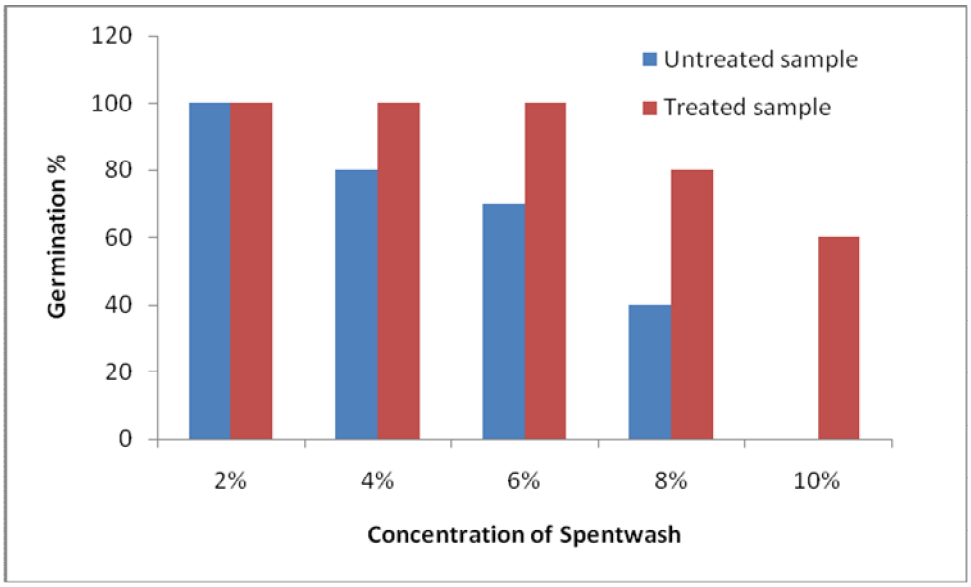

Fig 11: Effects of various concentrations of spent wash on germination of *Phaseolus mungo* L.

There was 60% seed germination at 6 % (v/v) concentration of spent wash after 72 hours. But, after bacterial treatment, 100% seed germination was recorded at 6% (v/v) concentration. In the bacterial treated sample, 80% and 60 % germination were recorded at 8% and 10% (v/v) concentration respectively, which was higher than with the untreated sample. The higher percentage of seed germination in bacterial decolourised spent wash samples might be due to degradation of organic compounds which caused adverse effect on seed germination [20, 21, 35, 37].

CONCLUSION

Bacillus coagulans ABS012 showed the maximum decolourization (44.30 %) when cultivated at 35^0 C for 5 days using modified spent wash medium containing 1% (w/v), glucose; 0.3 % (w/v), peptone; 0.1% (w/v), yeast extract; 0.2% (w/v), K_2HPO_4; 0.1% (w/v), KH_2PO_4; 0.05% (w/v), $MgSO_4$; 0.01% (w/v), $NaNO_3$; 10 % (v/v), distillery spent wash; pH-6.0 under static condition. The HPLC analysis confirmed the biodegradation of colour containing compounds of spent wash by the bacterial isolate. The toxicity reduction after bacterial decolourisation confirmed the reduction of toxic compounds from the spent wash.

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