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Decolourization of Molasses Based Distillery Waste Water by Using Calcium-alginate Immobilized Fungal Strain

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Abstract: Decolorization of intensely brown colored distillery wastewater was carried out by *Fusarium moniliforme*, fungus isolated from distillery wastewater. Under optimal performance parameter, (supplementation of glucose 0.8 %, w/v, NH_4NO_3 0.03 %, w/v, pH 5.0 and temp 30°C) isolated fungal strain showed maximum decolourization of almost 80 % in 6-7 days. Further, attempts were undertaken to improve the process of decolorization of distillery wastewater by the whole cell immobilization of isolated fungal strain *Fusarium moniliforme* in calcium alginate in indigenously designed bioreactor. In case of single stage bioreactor the immobilized cells showed 76-77 %, decolorization, a 3-4 % lower than maximum declorization attained by free cells. However, retention time for decolorization of waste water was one day shorter. The immobilized cells were used in 5 repeated treatment cycle of 5 days each without any significant loss in decolorization potential. Using the two stage bioreactor system for decolorization of distillery wastewater, there obtained the further decrease in acclimatization period by another one day i.e. the process was completed in 4 days with almost similar decolourization capability.

Key words: Industrial pollutants, Bioremediation, decolourization, Distillery waste water.

Introduction

Distillery waste water is perceived as one of the serious pollution problems of the countries producing alcohol from the fermentation and subsequent distillation of sugarcane molasses. According to an estimate, there are 315 distilleries in India. In which 2.7 billion liter of alcohol is produced, generating 40 billion liters of waste water annually and rated as one of the 17 most polluting industries [1,2,3]. Distillery waste water is characterized as one of the caramelized and recalcitrant wastes containing extremely high COD, BOD, inorganic solids, color and low in pH. Digested molasses distillery waste water cannot be decomposed by the usual biological treatment processes because of its content of melanoidin; a polymer formed by the amino-carbonyl reaction in food processing and preservation processes. Although highly distributed in nature this polymer is not easily degraded by micro-organism. Not only melanoidin, some other recalcitrant's compounds are also present in the waste water such as caramel. The unpleasant odor of the waste water is due to the presence of skittle, indole and other sulphur compounds, which are not decomposed by yeast during fermentation [4], resulting, color problems, slime growth, thermal impact, scum formation and loss of aesthetic beauty in the environment. They also increase the amount of toxic substance in the water, causing death to the

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flora and fauna as well as affecting the terrestrial ecosystem [5].

Several emerging technologies such as electrochemical destruction, photocatalysis and sorption are promising for molasses wastewater decolorization [6,7]. However, these approaches often involved complicated procedures and economically unfeasible. The conventional treatment process i.e. aerobic and anaerobic treatment process can be accomplished but only low removal of melanoidins and COD is still higher than the standard permissible value of different agencies. Therefore, there are still demands to develop innovative biological methods capable of providing amore clean up of the pollutant in more economic fashion. Microbial decolorization is an environmental friendly and cost competitive alternative to chemical decomposition process [8,9]. There are a number of fungi which play an important role in the decolorization of distillery effluent such as *Penicillium decumbens,* A*spergillus sp., Aspergillus niger, Aspergillus niveus, Flavadon flavus, Cladosporium caladpsporiodes* [10,11,12,13], *w*hite rot fungi *(Phanerochaete sp., Pleurotus sp., Trametes versicolor, Coriolus sp.*) [14,15,16].

Pure fungal cultures have been studied in order to develop bioprocess for decolorization in molasses wastewater. However, performace of fungal decolorization have limitations due to the presence of long growth cycle and spore formation [14,15].

In recent years immobilized biocatalyst technology has rapidly emerged as a novel means to utilize enzymes and whole cells as efficient and heterogeneous biocatalyst for a multitude of industrial applications [17]. Moreover, using biocatalyst, the process might be controlled in a better and easier than conventional treatment methods with free cells. There is also another merit that immobilized cells are more stable than free cells and exhibit totally different hydrodynamic characteristics than surrounding environment[18]. Various types of solid matrices like polyacrylamide gel, alginate, carrageenan, agarose, porous glass, plastic beads, activated carbon, diatomaceous earth, cement balls, chitosans, chitins, etc.[19]. In the recent years, the immobilization of biocatalysts with polyvalent salts of alginic acid has received much attention because of low cost of alginate and the mild conditions of immobilization [20.] Therefore, in the present study the effect of operation parameters on distillery wastewater decolorization by Ca-alginate immobilized isolated fungal strain *Fusarium moniliforme* in indigenously designed bioreactor were investigated.

Material and Methods

Collection of sample

The raw wastewater sample was collected from the local distillery of Dehradun, India where the raw distillery waste water was generated and discharged for further treatment. The raw waste water was very hot (almost 90^{o}C temperatures) collected in propylene container and cooled to room temperature. The digested waste water was collected from its bio-methanation anaerobic digester plant and storage lagoons. The waste water was stored in dark at low temperature under refrigeration before being used as substrate for biological treatment.

Microbial strain

The fungal strain was isolated from the soil of the anaerobic digester of the treatment plant by enrichment culture technique [21]. The petriplates were incubated at 30^{o}C for 5 days for the growth of the culture. Plates showing some decolorization were subculture repeatedly. Spread plate technique and Streak plate technique on agar media containing 10 % (v/v) digested wastewater supplemented in agar as sole source of carbon and nitrogen was used for isolation of microbial culture. The pure cultures obtained were maintained on potato dextrose agar (PDA) slants and sub cultured every fortnight. The fungal isolates showing the better decolorization potential were further characterized based on their various morphological parameters such as color, diameter of the mycelia and microscopic observation of spore formation and in conjunction with Gilman's manual of soil fungi (Volume- II) identified as *Fusarium moniliforme* and designated as *Fusarium moniliforme* NG_4 was found to possess a high potential of decolorizing digested distillery waste water up to 40 % (v/v) in minimal salt media was used in all the decolorization/treatment studies [22]. The different operating parameters i.e. pH,

temperature, substrate concentration: carbon & nitrogen for the treatment studies optimized with free cell culture were used for immobilization studies.

Immobilization of fungal isolate by sodium alginate entrapment

Fungal isolate from slants were sub cultured onto petriplates containing mineral salt media supplemented with 10 % (v/v) of digested distillery effluent and incubated at 30°C for 5 days. The spores were asceptically harvested in sterilled distilled water (10^7-10^8 spores / ml). To 400 ml of 8 % (w/v) Na- alginate solution, previously autoclaved at 110°C for 10 min and cooled to room temperature, was added 600 ml of spore suspension containing nearly 10^7-10^8 spores/ml. The contents were mixed thoroughly and the mixture was poured drop by drop into 1 % (w/v) sterilized solution of $CaCl_2$ with the help of an injection syringe, at 10°C, under aseptic conditions. The beads thus formed were allowed to harden for an hour at 10°C. Beads were then washed thoroughly with 0.9 % NaCl solution and stored at 4°C before use [23].

Single stage bioreactor

The immobilized fungal isolates were loosely packed in an indigenously designed bioreactor (Fig. 1) which was connected to a water suction pump through a side arm containing sterilized glass wool to circulate air into the fermentor at the rate

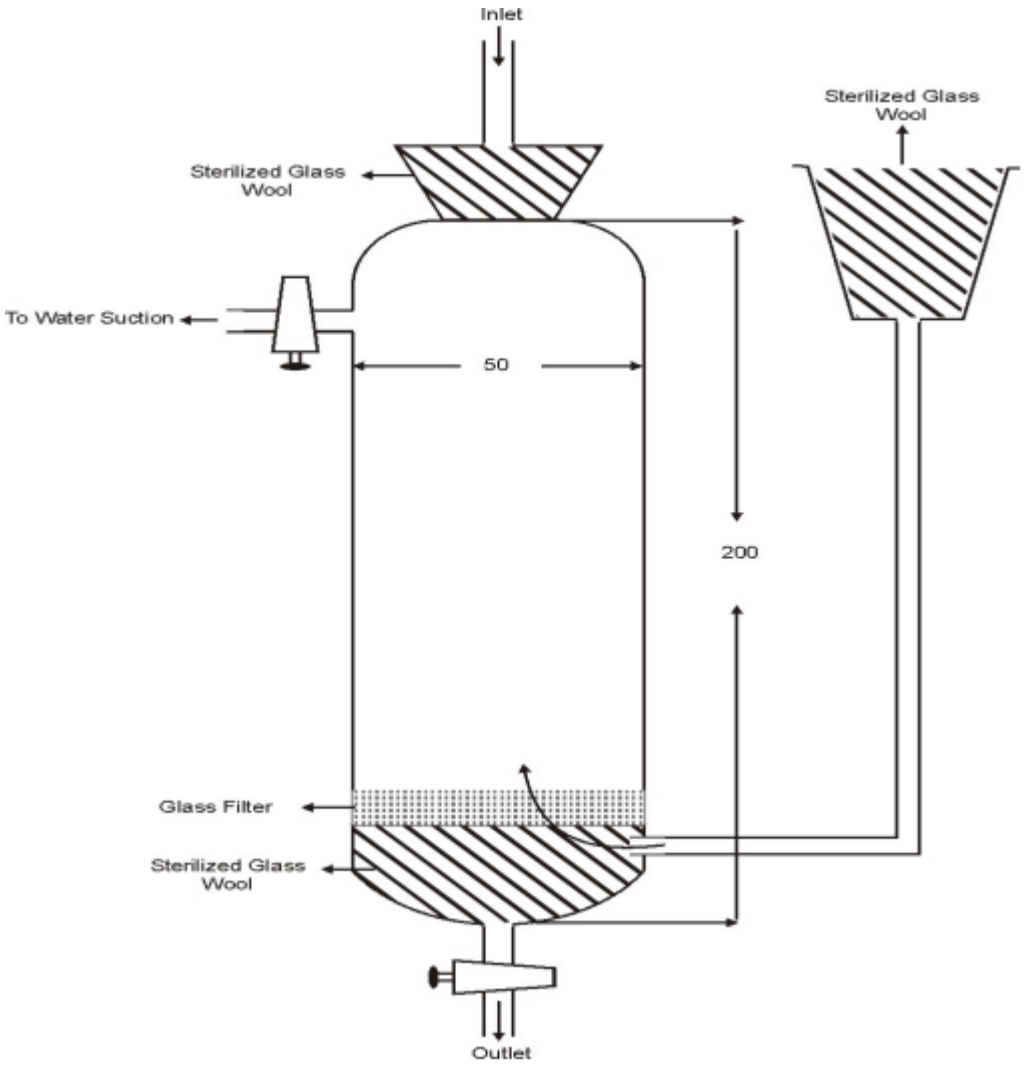

Figure 1. Single stage bioreactor

of 2 l/min. Decolorization was carried out using the replacement batch culture method. The decolorization potential, BOD and COD was monitored every 24 h. The suction of the sterilized air from the bottom of the reactor also provided an automatic stirring and mixing of the immobilized cells with the medium.

Two stage bioreactor

A two stage bioreactor was also designed which is essentially a combination of a two single stage bioreactor in series. The working of the system is the same as that of single stage bioreactor. The outlet of the first reactor was connected to the inlet of the second bioreactor. The treatment of digested distillery effluent was carried out using the replacement method, using immobilized whole cells of fungal isolates. In this way a continuous two cycle replacement for the treatment of digested distillery effluent was achieved. The decolorization potential, COD, BOD was monitored every 24 h (Fig. 2).

Decolorization assay

The decolorization of digested distillery waste water measured as a decrease in optical density of supernatant of treated medium at 475 nm against uninoculated waste water medium maintained aseptically as control and expressed as the percentage decrease in absorption. Experiments were performed in triplicate and samples were withdrawn at regular intervals for determination of decolorization.

Color unit = Estimated color x dilution factor.

Analytical analysis

Analysis to determine the COD,BOD, specific gravity, total reducing sugar, total sugar, carbon,

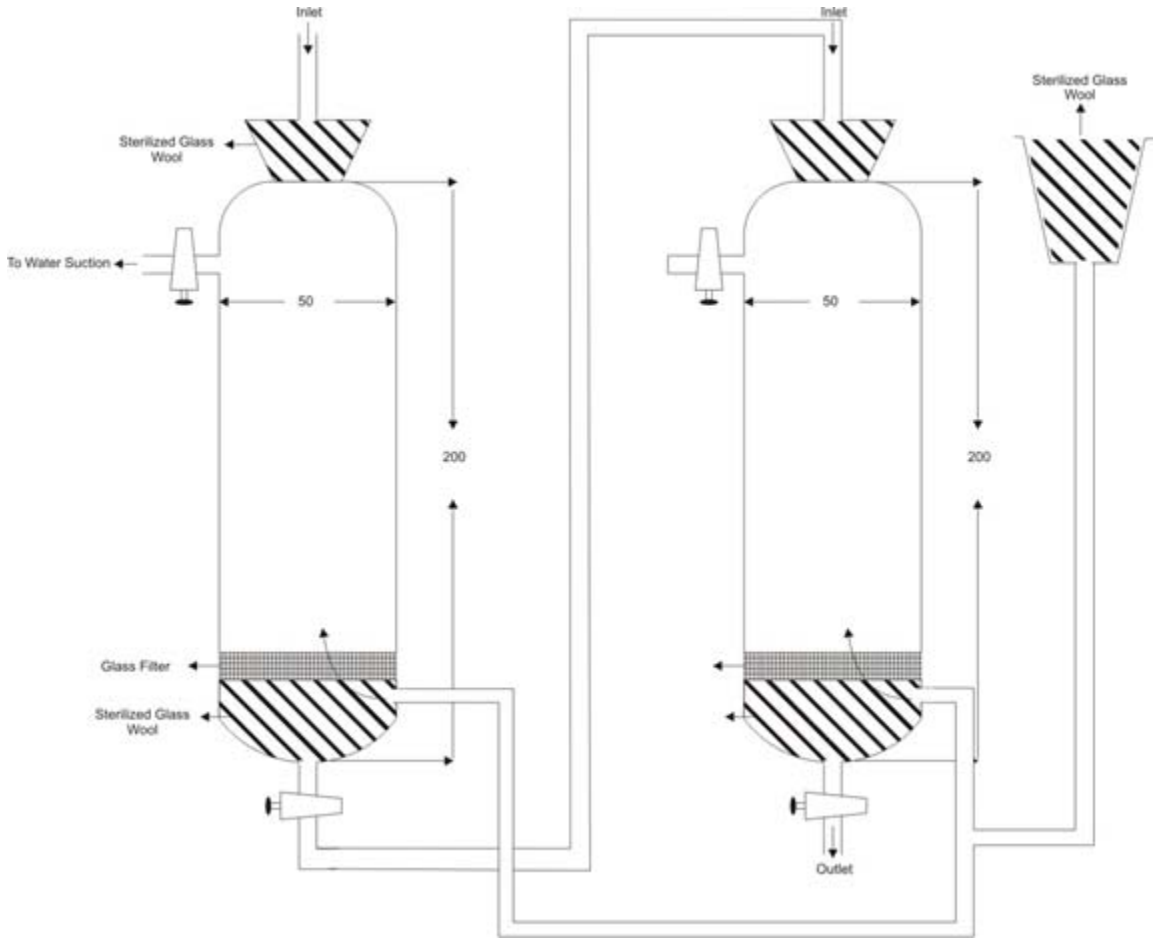

Figure 2. Two stage bioreactor

nitrogen, phosphorus, electrical conductivity etc for digested waste water were those described in APHA[24].

Results and Discussion

Time course study of decolorization and COD removal at optimum performance parameters

Under optimal performance parameter, supplementation of glucose 0.8 % (w/v), NH_4NO_3 0.03% (w/v), pH 5.0 and temp 30°C. The results showed the increase in the biomass accumulation with corresponding decrease in the total sugar concentration between 0 to 4 days of incubation. The percentage decolorization and COD reduction in the same period shows a slow trend. The rate of microbial activity of decolorization enhanced between 4 to 8 days of incubation. Lastly, the rate of decolorization slowed down between 8-12 days of incubation (Fig 3). The digested distillery effluent contained little, if any, readily available carbon source inspite of its high sugar content. The supplemented readily available carbon source depleted within two days of incubation. Similar, results for optimization of decolorization of distillery wastewater by different microbial cultures were reported by the previous workers [9,11,15,21]. The above results indicate that the digested distillery waste water contained little readily available carbon source so supplementation of basal media readily available carbon source was necessary for metabolism of microbe in digested distilleries waste water. However, at lower concentration of digested distillery waste water the growth of fungal strain was supported by the available carbon source, but as the concentration of digested distillery waste water in basal media increase, simultaneously the concentration of inhibitory effect also increases, which affect the growth of fungal strain. Therefore, at high concentration to support the growth of fungal strain supplementation of basal media by readily available carbon source was necessary to support the growth of fungus. Also addition of secondary carbon sources enhanced the degradation of xenobiotic compounds [11,25,26]. It is due to certain enzyme system as speculated by previous workers [10,27,28], confirmed by the enzymatic studies

It represents optimum growth conditions for the fungal culture, which may possibly reflect the optimum activity of enzyme responsible for carrying out. Supplementation of basal media was

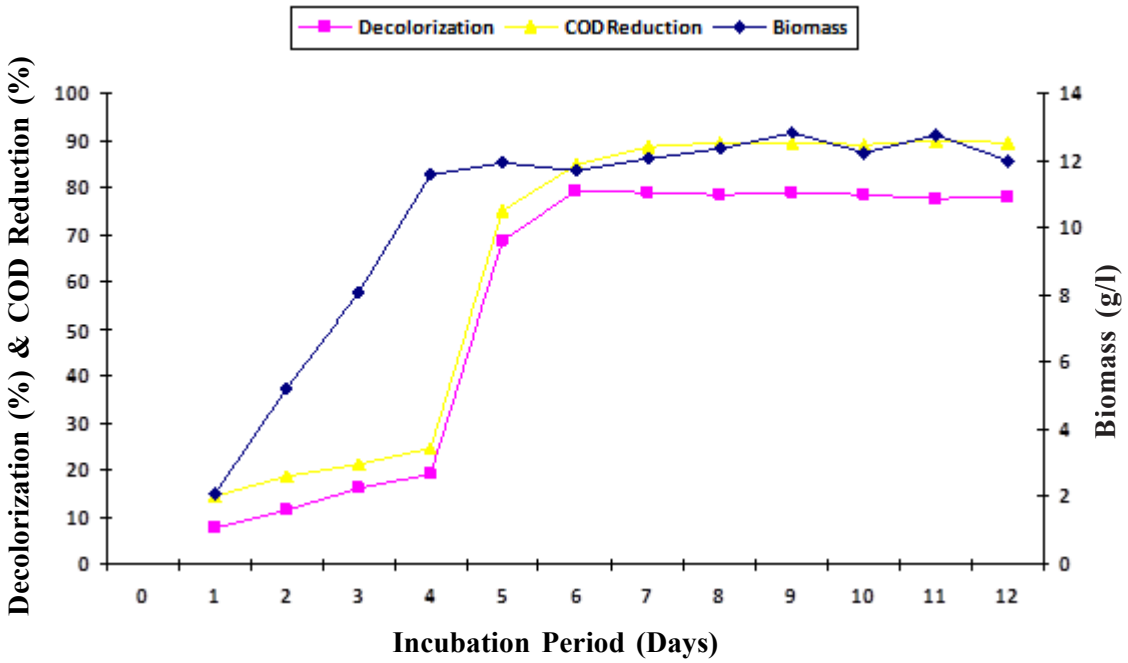

Figure 3. Time course study of biomass, decolorization and COD removal at optimum performance parameters

necessary to support the growth of fungus. Depletion of this supplemented carbon source in early stage of incubation may allow the fungus, drift to refractile carbon of the waste water for further growth. This co-relates the fact that the process of decolorization and growth of fungus to a certain extent depends on the uptake of the non-readily available carbon source, melanoidin component of the digested distillery waste water. The maximum decolorization of 79 % was attained within 6-7 days of culture incubation. There was no further significant color removal observed after 7th day of incubation. However, increase in cell mass was at its maximum value on 4th day of culture incubation which gradually becomes stationary after 4th of incubation and showed a gradual decline in growth rate after 6th day of incubation. COD reduction showed a continuous upward trend and reaches the peak value of reduction on 8th day of culture incubation. After the 8th day of culture incubation, there was no further substantial decrease in the COD reduction and the culture entered the declining stage of the growth phase. The process decolourization of distillery wastewater was carried out for the 12 days, though, no substantial declorization was observed after 6 days of culture incubation. From the results, it seems that the time course data on the microbial activity of decolorization confirm to the classical scheme of Gaden [29]. The process of decolorization is considered as Type-II fermentation processes, characterized by two rate maxima with regard to the growth of the culture and decolorization of the digested distillery waste water [29].

Decolorization of digested waste water as a function of innoculum level

The innoculum level was found to have a profound effect on decolorization, using immobilized cells, unlike free cell treatment (Fig 4). The maximum decolorization potential of the fungal isolate was obtained that at 20 % (w/v) innoculum level. Higher level of innoculum was found to be much inferior for decolorization potential. Perhaps, a high concentration of inoculum hinders the aeration, agitation process and also the diffusion characteristic across the immobilized bed.

Decolorization as a function of temperature

The optimum temperature for maximum decolorization performed in immobilized was found to be 32°C with an optimum performance range of (30-45°C) (Fig 5). This temperature is slightly higher than the optimum range of temperature obtained with free cells treatment process. It is expected that large scale trials, the temperature increases 3-4°C due to microbial

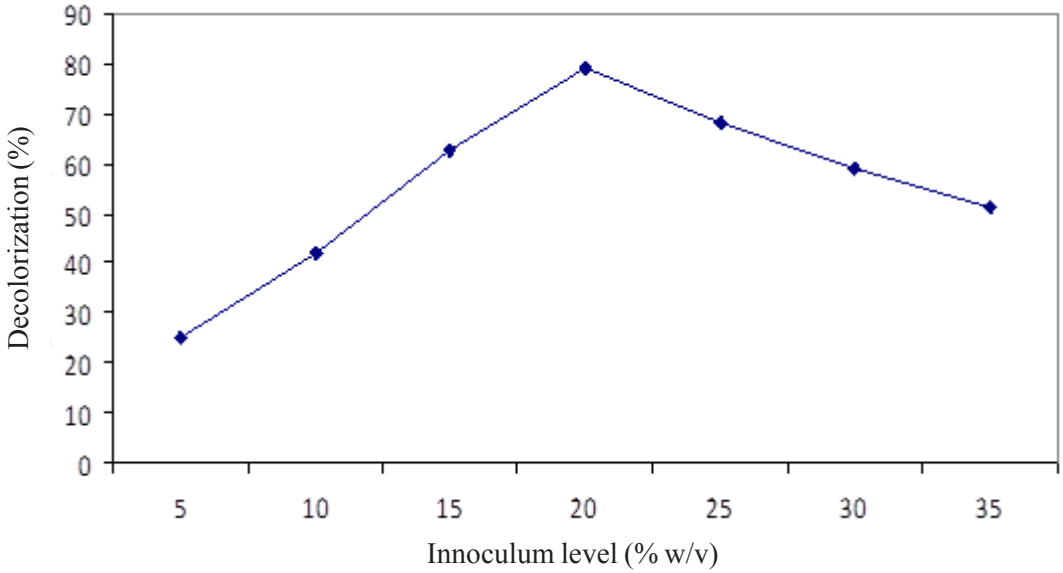

Figure 4. Effect of different innoculum levels on decolorization of digested distillery effluent by immobilized cells of *Fusarium moniliforme*

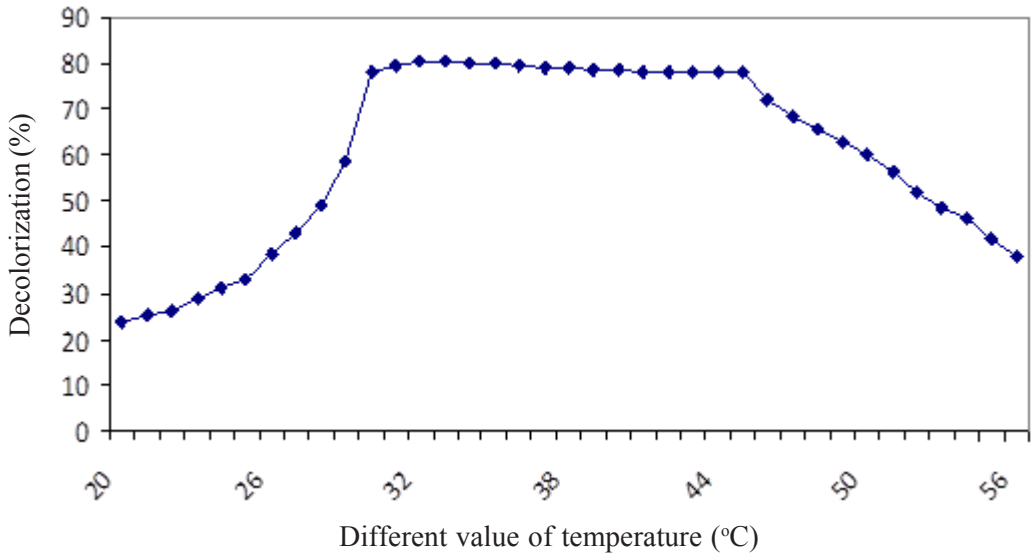

Figure 5. Effect of temperature on decolorization of digested distillery effluent by the immobilized cells

activity, therefore tolerant microorganisms may survive better [33]. The isolated fungal strain in the present study is thermotolerant, hence suitable for immobilization in calcium alginate.

Time course of decolorization with immobilized cells

The immobilized cells have been used in a variety of applications such as biotransformation, biosensors, production of ethanol, degradation of phenols, degradation of distillery wastewater etc.[30,31,32,33]. Among the different immobilization methods, gel entrapment is the most common. Entrapment of cells in calcium alginate is one of the most simplest, cheapest and non-toxic that most frequently method of immobilization [34]. In the present study decolorization of digested distillery waste water by calcium alginate immobilized cells of *Fusarium moniliforme* NG_4 was investigated. A slight change in the performance parameters was observed. The most significant change was broad range of optimum temperature (32°C-45°C) (Fig 4) than the free cell (28°C-30°C). It was not unexpected since immobilization is known to bring about certain physical and physiological stress on the cell [21].

The time course for degradation of melanoidin pigment; decolorization of digested distillery effluent using immobilized cells is shown in (Fig 6). The result show that in the case of Ca-alginate immobilized cells 6 days period is required for apparently complete decolorization with the maximum decolorization of 78 %. From the figure 6, it was observed that decolorization increase sharply up to 6 days of treatment which tapers at 7th day and then after it reached a steady state at which no further increase was observed even by increasing the incubation time. However, it results in decrease in COD of digested distillery effluent up to 10 days of incubation. However, it can be seems that after a three days of initial lag phase, the decolorization increase rather sharply, indicating that the immobilized cells, like free cells, require 5 days for proper maturation and acclimatization for efficient decolorization of digested distillery effluent, degradation of melanoidin under optimum condition of temperature, pH, substrate concentration and aeration.

The decolorization with immobilized cells was slightly lower than free cells. This lowering in decolorization capability of the cell upon immobilization might be due to the various physical and environmental stress conditions prevailing, which prevents the cells from interacting freely with the environment. It is already known that half life of cells is extended upon immobilization, as growth of these cells is greatly retarded due to environmental stress, so that these cells can be maintained

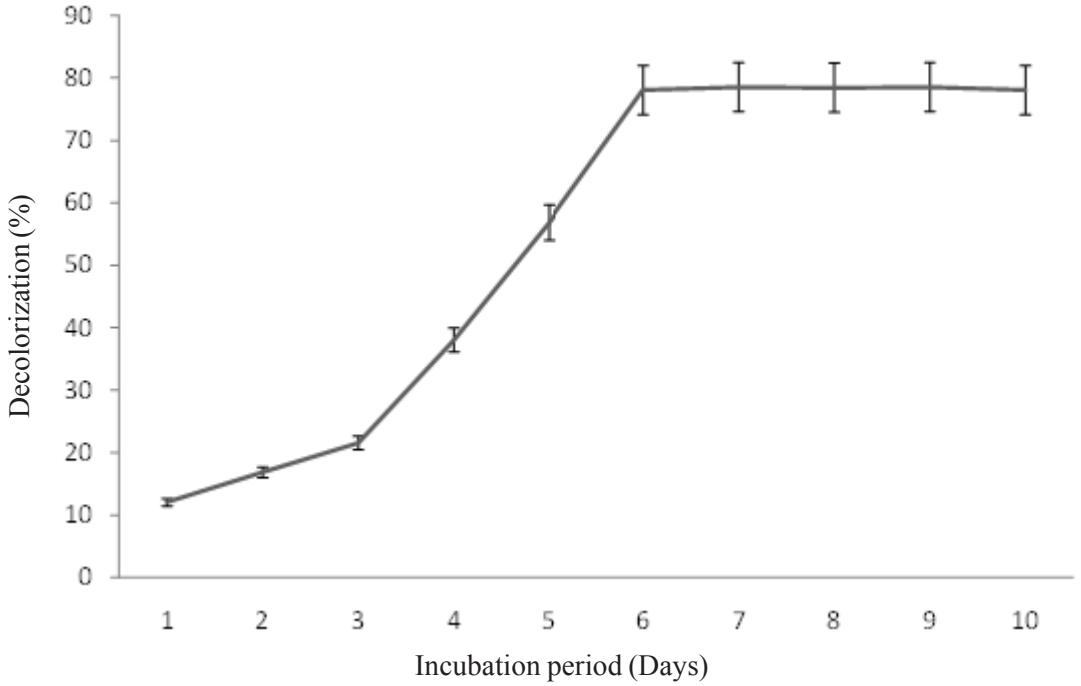

Figure 6. Time course profile of decolorization of digested distillery effluent by Ca-alginate immobilized cells of *Fusarium moniliforme*

on minimum requirement for a longer period [23,30,31,32]. The immobilized cells took nearly a lag phase of 3 days and 6 days before reaching steady state of maximum decolorization (Fig 6). Probably during acclimatization these immobilized cells are activated properly oriented to face the changed environment from free to restricted interaction with the waste water. In addition, the result suggested that the immobilized cells after proper acclimatization may be used in subsequent repeated cycles. In view of the above, immobilized cells were packed in a vertical column bioreactor (Fig 2 & 3) and treatment was carried out in a batch and repeated batch process.

Use of immobilized cell for repeated treatment cycle

From the results obtained in preceding system, it is apparent, that the immobilized cells may be used with the fresh wastewater after the 5-6 day acclimatization of immobilized cells that would give better decolorization. Experiment was conducted in which Ca-alginate were acclimatized for 5 days in 50 % (v/v) digested distillery effluent in minimal media under optimum condition in a fabricated single stage bioreactor. The decolorized waste water was then replaced by the fresh digested distillery effluent and the decolorization potential of strain was monitored daily at 24h interval. It was found that 75-78 % decolorization was attained during the 5-6 days of acclimatization period. The similar results were obtained during the second, third, fourth and fifth cycle, each requiring 5 days of culture incubation. In other words, after the acclimatization of immobilized cells for 5-6 days, the same batch of immobilized cells was used in 5 repeated treatment cycle without any significant loss in decolorization potential (Fig 7). An approach was used for decolorization using a two stage bioreactor for decolorization of digested distillery effluent using Ca-alginate immobilized cells. The digested distillery effluent after 2 days of holding time of acclimatization in first bioreactor was transfer to second stage bioreactor (pre-acclimatized immobilized cells). As expected, a two stage bioreactor gave a better yield. The important point is that the decolorization of 75 % was obtained within 4 days of culture incubation. Further increase in retention time does not have any

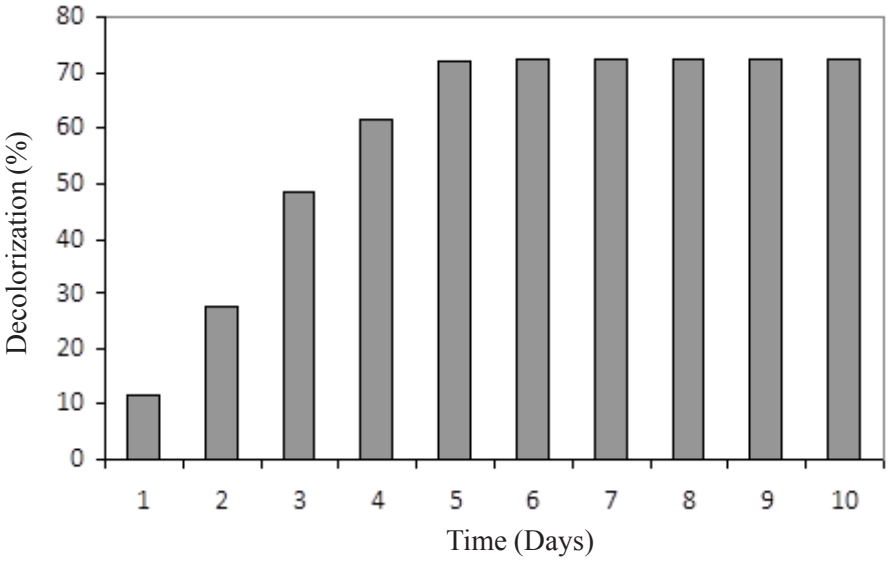

Figure 7. Decolorization of digested distillery effluent by using immobilized fungal cells in single stage bioreactor

influence on the decolorization capability of the fungal cells employed in two stage bioreactor process (Fig 8). The results obtained in single stage bioreactor process showed that decolorization was 3-4 % lower and retention time for decolorization of waste water was one day shorter than free cell treatment process. Nevertheless, the use of immobilized cells for decolorization of digested distillery waste water seems promising. However, possibility of using Ca-alginate immobilized cells in continuous decolorization process is doubtful because of the long period (5-6 days) required for the decolorization even after proper acclimatization of cells. The process could be performed effectively up to 5 repeated cycles without any significant loss in decolorization potential of the strain in single stage bioreactor. This may further add up to the feasibility of the process for a longer period of time which can be exploited at an industrial scale after establishing

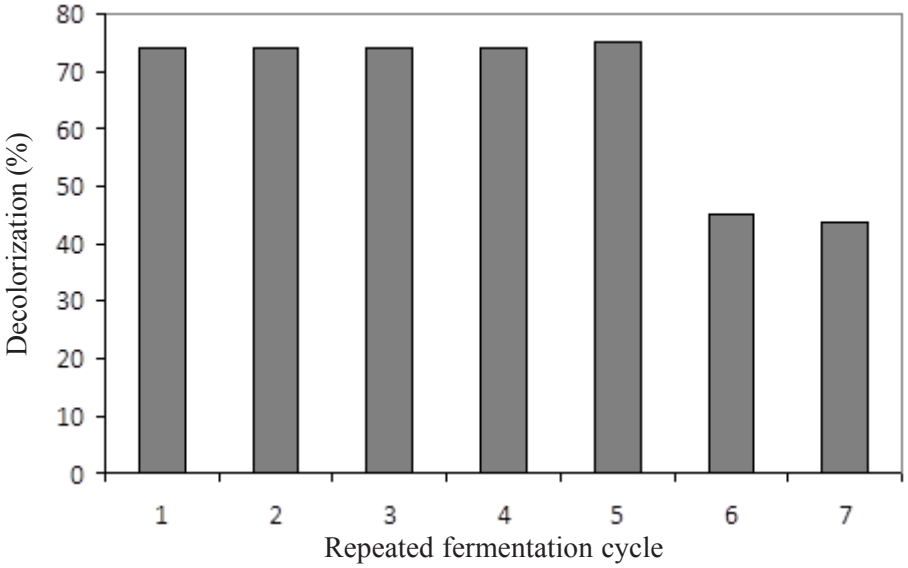

Figure 8. Decolorization of digested distillery effluent by using immobilized fungal cell in repeated fermentation cycle

the technical problem. In the sixth repeated cycle the loss in decolorization capability was almost 50 %, which was really the subject of concern. Further, using the two stage bioreactor system for decolorization of digested distillery waste water, the system further decrease the acclimatization period by another one day i.e. 75 % decolorization was observed within 4 days of culture incubation. This system was maintained up to 20 days without any significant loss in decolorization capability.

These results are in agreement with results of the previous workers employing different strains of fungi under free and immobilized conditions for decolourization of distillery wastewater [9,11,26,27]. The level of decolorization capability of the different fungi varies between 30-90 %, in 8-30 days of culture incubation where, the process were only capable of decolorizing 10-30 % (v/v) diluted digested distillery effluent [10,11,14,16,35,36].

However, in the present study, our results showed about 78-80 % color removal and almost 85-90 % COD removal of highly recalcitrant digested distillery waste water using indigenously isolated fungi at a higher concentration (50 %,v/v) in 4-5 days of culture incubation.

To the best of our knowledge, this is a first report of a high degree of decolorization achieved at high concentration of wastewater (50 %, v/v) within a short time. However, comparing the decolorization activity of different fungi is not appropriate, as the conditions employed by the various workers are not the same. Further our culture was equally effective in decolorizing raw distillery waste water and digested distillery waste water collected either after anaerobic treatment or aerobic treatment. This shows that changes in melanoidin pigment in raw or during aerobic and anaerobic treatments does not have any effect on decolorization capability of the fungal strain *F. moniliforme*NG_4.

Conclusion

In recent years immobilized biocatalyst technology is giving popularity in industrial processes and efforts are being made to produce commercially important products by fermentation and design bioremediation process by immobilized whole cells. The immobilization of fungal cells in batch and repeated batch process is better than free cell culture. The main aim of immobilization for was done at scale for a long time without preparing inoculums. The sodium alginate used as a solid matrix provide sufficient oxygen as well as microbial film forming capability into the medium simultaneously to achieve higher decolorization at constant rate in short interval of time. Among the immobilized cell bioreactors, no doubt that batch and repeated batch immobilized is an efficient one which can be adopted for the treatment of distillery wastewater containing melanoidin compounds. A proper choice of immobilized culture, careful consideration of various design parameters will make treatment process cost effective in long run. Though, there is a great potential for immobilized whole cells to be used in bioreactor for decolorization of digested distillery waste water. Further research will, however, be required to develop it into commercial reality.

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