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Comparative study on treatment of cassava wastewater using free, immobilized and biofilm of *Trichoderma harzianum-Trichoderma viride* consortium

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ABSTRACT

Cassava (*Manihot esculenta*) being rich in starch is one of the critical industries in the agro-industrial sector. The present study aims to do a comparative study on the treatment of cassava wastewater using biological and eco-friendly treatment approaches consisting of native bacterial species and fungal consortium in free, immobilized and biofilm batch mode. Studies were performed using the mixed culture of native bacteria, and the results were compared with that of a mixed fungal consortium of *Trichoderma harzianum* and *Trichoderma viride* which were known for its cyanide degrading efficiency and reducing COD burden. Cyanide and COD were two critical pollutants for the study. The effect of treatment time on cyanide and COD removal was investigated without adding any buffer to the wastewater. The maximum cyanide and COD removal efficiency for wastewater by using mixed native bacterial were 41.12% and 53.07% respectively. The removal efficiency by mixed bacterial culture was not too high owing to the low pH of cassava wastewater. Even in the low pH of cassava wastewater the maximum cyanide and COD removal efficiency using fungal consortium were 63.77% and 74.3% respectively. The growth pattern of fungal consortium reveals that six days treatment period showed maximum biomass of 3.721 g/L of dry weight. Thus, six days of treatment of fungal consortium can be used for the treatment of cassava wastewater. Furthermore, the optimized value of temperature and inoculums dosage for fungal consortium was found to be 30°C and 4% (v/v) of wastewater. Under optimized condition free, immobilized and biofilm batch reactors were setup. Biofilm batch reactor gave COD removal efficiency of 88% followed by suspension with 83.5% and least in an immobilized batch reactor with 76.5%. Cyanide removal efficiencies of 98.19%, 77%, and 87% were obtained in a biofilm, suspension and immobilized system respectively. The experimental results showed that final pH after treatment in all three systems was in dischargeable limits. The study revealed that the biofilm batch reactor which gave the highest removal efficiency could be considered as the best method for treatment of cassava wastewater.

Keywords: Manihot esculenta; Cyanide; Consortium; *Trichoderma harzianum*; *Trichoderma viride*.

1. Introduction

Water pollution is an undesirable change in the physical, chemical and biological properties of water that has a harmful effect on living things is water pollution. Agro-industrial processes generate large volumes of wastewater and solid residues whose quality varies greatly. Generally, farm-produce processing activities use abundant water to wash and treat products, at which point the water is loaded with harmful elements and compounds [1]. Tapioca cassava (*Manihot esculenta*) is an essential source of starch and also serves

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as the staple food. In India, nearly 60% of cassava is used industrially in the production of sago, starch and dry chips [2]. Nearly 500 sago producing units are located in Tamil Nadu of which about 470 units are located in Salem district, India. 90% of the cassava starch produced in India is from Tamil Nadu while the remaining quantity is from Kerala and Andhra Pradesh. Intensive cultivation practices resulted in the mean yields for cassava of 34.37 ton/ha in India [3]. The cassava tubers are the raw material, and it is converted into commercial sago through indigenous technology. During the process, on an average from 30,000 to 40,000 L of effluent is generated per tonne of sago processed and it takes about ten days for the water to be let out of the factory as effluent [4,5]. Linamarin and lotaustralin, are the two different cyanogenic glycosides in cassava plant. Roots and leaves contain the highest amount of linamarin (80%). Linamarin produces the toxic compound hydrogen cyanide (HCN) through enzymatic hydrolysis which can be hazardous to the consumer [6]. HCN is released during peeling, slicing and crushing often found in the wastewater discharges [7]. The detected cyanide level in cassava wastewater ranged between 10.4 and 274 mg/L depending upon the cyanogen glycoside content of the cassava varieties [8]. The production and consequent consumption of cassava have increased extensively in recent times. This increased utilization of processed cassava products has equally increased the environmental pollution associated with the disposal of the effluents [9,10].

Biogas recovery from the cassava wastewater treatment system has shown great potential for cassava processors using tarpaulin cover over the conventional anaerobic lagoons and utilizing the biogas for roasting of sago and or for generation of electrical energy [11]. This method suffers from various drawbacks like its high requirement of buffers for adjusting the pH of wastewater. Also, methanogenic bacteria are susceptible to the high cyanide concentration. Alkaline chlorination is also effective at treating cyanide to low levels, but cyanogen chloride may evolve as a relatively toxic gas [12]. Therefore, there is a need for an improved technique to resolve the problems like cost-effectiveness, minimization of buffer required, etc. Among the microbes, fungi are more tolerant to high concentrations of polluting chemicals than bacteria. The fungal treatment system converts the wastewater organics into highly dewaterable fungal biomass [13]. Fungi and bacteria have also been known for their cyanide degradation property which converts cyanide to nontoxic compounds [8,14,15]. The present study aims in an eco-friendly and cost-effective method for treatment of cassava wastewater involving isolating, screening and generation of mixed culture of the native bacterial species and examining the feasibility of native bacterial culture and fungal consortium for the treatment of cassava wastewater in the natural pH conditions.

2. Materials and methods

2.1. Isolation, screening, and generation of the mixed bacterial culture from wastewater

Cassava wastewater was collected from Salem, Tamil Nadu. The value of total solids, total suspended solids, and total dissolved solids are analyzed by the gravimetric method. Cyanide in the wastewater was estimated by a modified ninhydrin method [16]. The wastewater was serially diluted with sterile saline water (0.85% NaCl). 1 ml of the wastewater from different dilutions which were made using saline water was plated on sterile nutrient agar in duplicates. Distinct bacterial colonies were inoculated in 250 mL conical flasks having 100 mL of sterile nutrient broth having one mM sodium cyanide. The inoculated conical flasks were kept in the incubator at 37°C at 110 rpm. The colonies resistant to cyanide were successfully screened for preparation of mixed bacterial culture. The mixed bacterial culture was obtained by inoculating the successfully screened colonies into the 250 mL conical flask containing 100 mL of sterile nutrient broth overnight at 37°C at 110 rpm. The mixed culture was a subculture for further experimental use, and a stock of mixed culture was prepared and refrigerated at 4°C. Fig. 1 presents the scheme of the methodology used in the present study to treat cassava wastewater through a microbial and fungal consortium.

The mixed bacterial culture obtained was used for the treatment of cassava wastewater. Inoculation was done from the mixed bacterial culture in five 250 mL conical flasks containing 100 mL of sterile wastewater inside the laminar air flow chamber. All inoculated flasks were kept in an incubator at 37°C and 110 rpm. Each day one conical flask was removed from the shaking incubator, and bacterial biomass could settle, and samples were collected for cyanide and COD estimation. The cyanide and COD removal efficiency (%) of the sample was calculated using Eq. (1).

$$\text{Removal efficiency } (\%) = \frac{(\text{Initial concentration Final concentration})}{\text{Initial concentration}} \quad (1)$$

2.2. Treatment of wastewater using mixed fungal culture

The mixed fungal cultures were prepared by inoculating *T. harzianum* and *T. viride* individually from fully grown Petri plates aseptically in YPD (Yeast Peptone Dextrose) broth and incubated at 37°C and is presented in Fig. 2a. The mixed fungal culture obtained was used for the treatment of cassava wastewater. Inoculation was done from the mixed fungal culture in five 250 mL conical flasks containing 100 mL of sterile wastewater inside the laminar air flow chamber. All inoculated flasks were kept in an incubator at 37°C and 110 rpm. Every second day one conical flask was removed from the shaking incubator and mixed fungal biomass was allowed to settle and samples were collected for cyanide and COD estimation. The removal efficiencies were calculated using the formula given in Eq. (1). Also, the biomass growth from the conical flask containing mixed fungal culture was estimated by passing the sample to preweighed Whatman filter paper in a Buchner funnel and then the filtered biomass along with Whatman filter paper was kept in an oven at 50°C overnight and then the weight was again measured. The biomass present was then estimated by subtracting the Whatman filter paper's weight from the final measured value. The study was conducted to investigate the effect of temperature on the treatment along with inoculum dosage for the COD removal from the wastewater. For the preliminary purpose the operating condition was chosen as pH 4.02 (native pH), temperature 37°C and inoculum dosage 1% (v/v) of cassava wastewater. To inves-

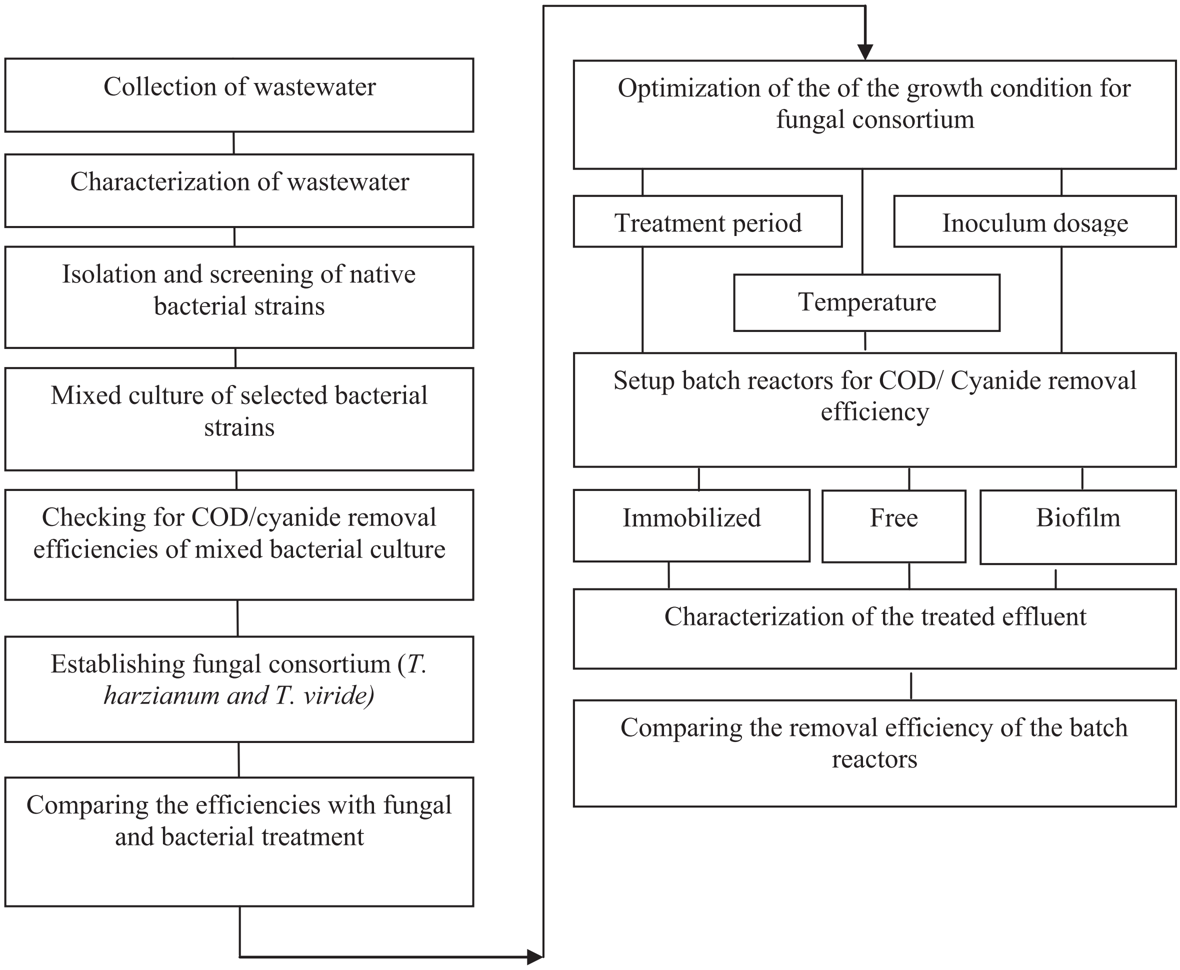

Fig. 1: Flow-chart of methodology.

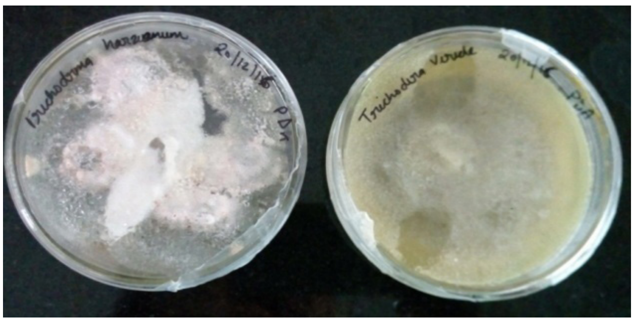

Fig. 2a. *T. harzianum* and *T. viride* cultures in PDA plates.

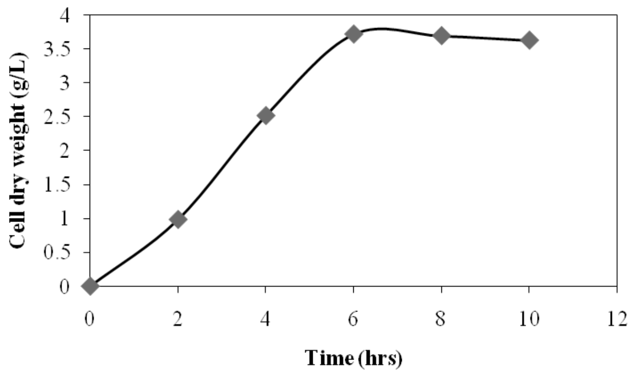

Fig. 2b. Growth pattern of fungal consortium in cassava wastewater.

tigate the effect each of operating parameters, each parameter was varied individually to obtain optimized condition while keeping other parameters constant.

2.3. Treatment using fungal consortium immobilized in calcium alginate beads

Cells were immobilized by entrapment in calcium alginate gel according to the method described in the literature [17]. A working volume of 400 mL was used for the experimental purpose. Calcium alginate beads containing 4% (v/v) of the fungal consortium was used at 30°C. The plastic support material was autoclaved before use to avoid cross-contamination. 4% (v/v) of the fungal consortium was used in cassava wastewater at 30°C. Samples were collected at an interval of two days for checking removal efficiency.

3. Results and discussion

3.1. Treatment of wastewater from mixed bacterial culture

The characteristic of the real wastewater was carried out by conducting basic laboratory experiment for the calculation of the concentration of different parameters like total solids, total dissolved solids, turbidity, pH, COD and more according to the given APHA standards. The result of the various experiment conducted to characterize the wastewater is tabulated in Table 1. Out of 11 isolated bacterial colonies,four colonies were selected after screening and mixed culture obtained from these colonies were used for checking cyanide and COD removal efficiency. The mixed bacterial culture treatment has a maximum cyanide removal efficiency of 41.12% as shown in Fig. 3a. During the initial 24 h, removal efficiency was only 3.92% which was not that much prominent. The increase in the removal efficiency after 24 h showed that the mixed bacterial culture got acclimatized in a wastewater environment and increased to 28.5% in 48 hrs. Cyanide degradation pattern illustrates that bacteria in the system are releasing cyanide degrading enzyme which is making them thrive in such wastewater [18]. Enzymes secreted by bacteria convert the cyanide into ammonia and CO_2 [8]. Thus, a slight increase in pH from 4.02 to 4.89 at the end of the treatment period is observed. From Fig. 3c it is seen that the maximum COD removal efficiency is 53.07%. After 24 h an increase in the removal efficiency is seen. Even with initial pH, 4.02 bacteria can grow in cassava wastewater by using starch as the substrate, and it also induced the amylase production so to breakdown the starch and used it as the carbon source [19]. After 96 h, the removal efficiency became almost constant showing stationary phase for the bacterial colony in wastewater. Though the continuous increase in removal efficiency is seen with the increase in treatment time, maximum removal efficiency is not very high because of the acidic nature of the wastewater as optimum pH for bacteria lies in the neutral range [20].

3.2. Treatment of wastewater from mixed fungal culture

3.2.1. Growth characteristics of fungal cultures developed from wastewater

Trichoderma sp. has been widely studied for its cyanide degradation properties [21] while *Trichoderma harzianum* has shown its ability to reduce COD from the cassava wastewater [19]. Therefore, strains of *Trichoderma harzianum*, *Trichoderma viride* were collected and used for the present study. The individual cultures took five days to grow in the Petri plates fully. Both the fungus showed radial growth in Petri plates. *T. harzianum* appeared white and reddish, and *T. viride* gave light greenish appearance in PDA plates depicted in Fig. 2a. The mixed culture of fungus in YPD broth took 3–4 days for full growth. The maximum biomass of 3.721 g/L of dry weight was seen on the 6th day shown in Fig. 2b. The exponential phase from 2nd to 6th day was seen. The stationary phase was seen after the 6th day, with almost constant biomass. Similarly, a study was conducted wherein researchers grew *Aspergillus oryzae* in natural rubber effluent [22] serum rich in COD and concluded that high COD removal is achieved with high fungal biomass.

3.2.2. Treatment of wastewater from a mixed fungal consortium

Maximum cyanide removal efficiency obtained is 63.77% as shown in Fig. 3b. As removal efficiency is higher in exponential phase (2–4 d) which indicates that cyanide removal is associated with the metabolic activity of Trichoderma consortium which is in agreement with previous studies [8]. The removal of cyanide is mainly due to enzymatic degradation of cyanide into formamide and subsequent production of ammonia [21]. Ammonia produced in the system was used as the nitrogen source by the fungal consortium, and that increased pH [23]. The pH increased from 4.02 to 8.14 in 10 d of treatment period which is also in correlation to the statement that fungi can alter the pH of the medium due to the differentiated transport of cation and anions during substrate transportation [20]. From Fig. 3d it is seen that the maximum COD removal efficiency by the fungal consortium is 74.3%. For the first 2d, the removal efficiency is 25% which exponentially increased to 60.5% and 74.3% on the 4^{th} day and 6^{th} day respectively. This period can be termed as an exponential phase because during this phase fungal biomass obtained was also high shown in Fig. 2b. Thus, higher biomass will use more starch as a carbon source from cassava wastewater and thereby reducing its COD. It was observed that COD removal efficiency is highest in 6 days of treatment and at the same time, the biomass was also highest (3.721 g/L). Thus, COD removal efficiency is directly related to the fungal biomass generated during the treatment period [24].

Table 1
Characteristics of untreated and treated cassava wastewater

Parameters	Untreated	Suspension	Immobilized	Biofilm
pH	4.02	8.38	8.44	8.53
Turbidity (NTU)	138	70.44	106	13.27
Total solids (mg/L)	2980	1693	2250	1200
Total dissolved solids (mg/L)	2650	1481	1989	1090
Total suspended solids (mg/L)	330	212	261	110
$BOD_{5,20}$ (mg/L)	1080	307	446	223
COD (mg/L)	1856	152.04	249.8	108.6
Cyanide (mg/L)	9.19	2.11	0.55	0.18

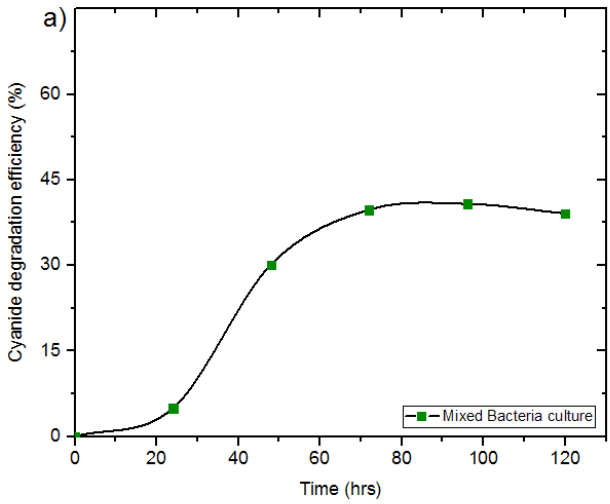

Fig. 3a. Cyanide removal efficiency using mixed bacterial culture.

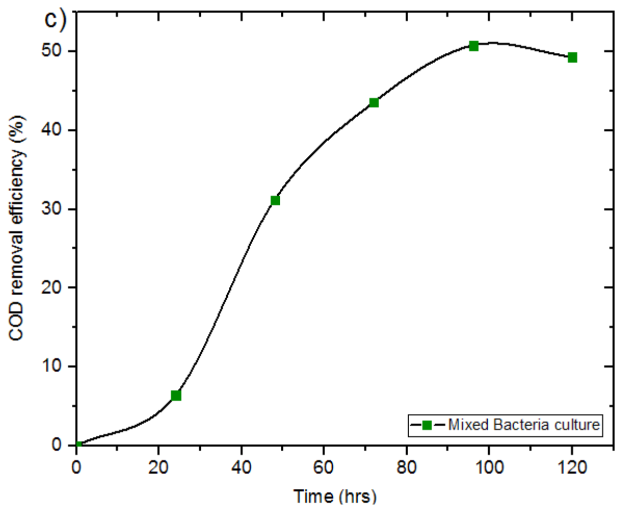

Fig. 3c. COD removal efficiency using mixed bacterial culture.

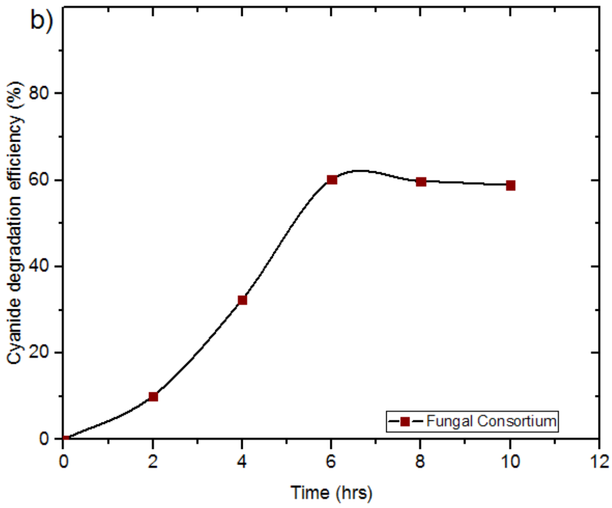

Fig. 3b. Cyanide removal efficiency using the fungal consortium.

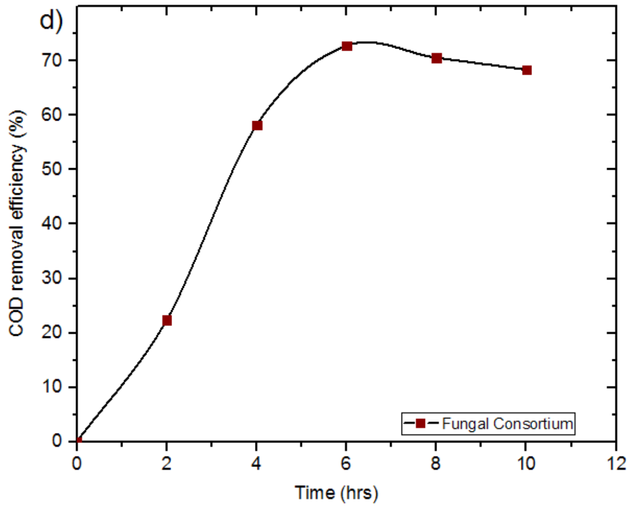

Fig. 3d. COD removal efficiency using the fungal consortium.

3.3. Optimization of growth parameters using the fungal consortium

3.3.1. Effect of incubation temperature on COD removal

In order to determine the optimum growth parameters using fungal consortium experiments were conducted to optimize temperature and inoculum dosage. The variation of removal efficiency with different incubation temperature was studied. Fig. 4a shows that at 20°C the consortium gave minimum COD removal. As the incubation temperature was increased from 20°C to 30°C the increase in the removal efficiency of COD from 65.3% to 79.7% was seen. Furthermore, the increment in incubation temperature from 30°C to 50°C showed a decrease in the removal efficiency of COD from 79.7% to 67.53%. This can be explained as, an increase in incubation temperature results in the growth of the fungus. Moreover, higher fungal biomass will able to consume more of the starch from the wastewater and thus reducing the COD value. However, after a specific temperature further increment in temperature did not support the growth of fungus because every organism has a specific range of temperature at which they grow properly. Since the incubation temperature of 30°C gave the highest removal efficiency, it is considered as optimum temperature for treatment of cassava wastewater using fungal consortium.

3.3.2. Effect of Inoculum dosage on COD removal

The effect of inoculum dosage of the fungal consortium was investigated by conducting a series of experiments, and the result are shown in Fig. 4b. While increasing the inoculum dosage from 1 mL to 5 mL the value of COD removal is 79.7% for 1 mL, 81.2% for 2 mL, 82.7% for 3 mL, 83.5% for 4 mL and 81.26% for 5 ml. This can be explained as, increase in the inoculum dosage results in the increase of COD

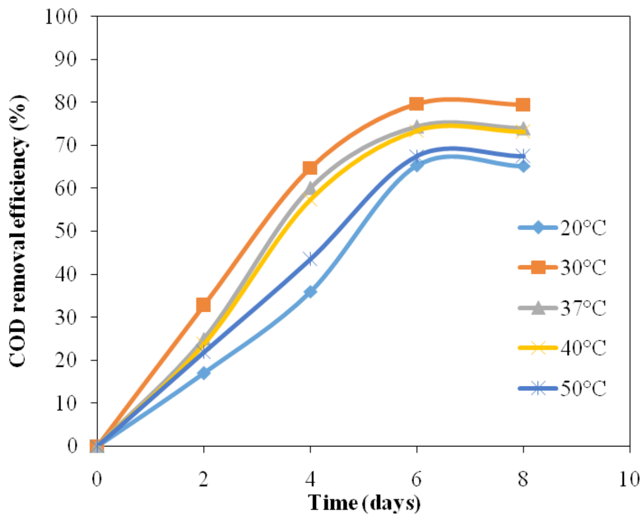

Fig. 4a. Effect of incubation temperature on COD removal.

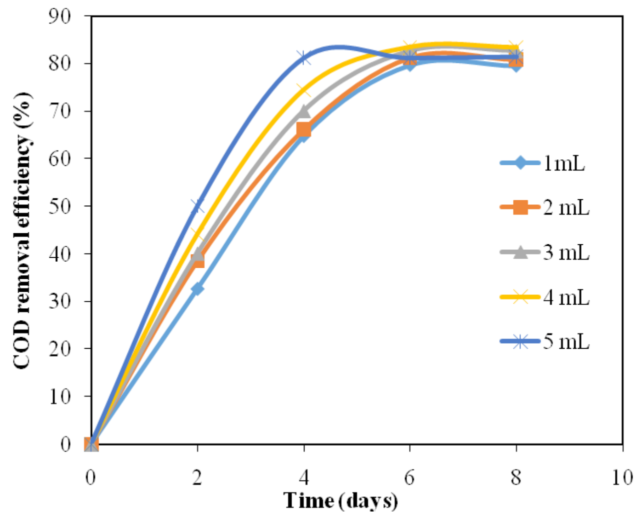

Fig. 4b. Effect of inoculum dosage on COD removal.

removal efficiency of the processes. Also with the increase in inoculum dosage, the lag phase was seen shorter, and fast growth rate is observed when inoculums dosage was increased from 4 mL to 5 mL [25]. Thus inoculum dosage of 4 mL can be considered as optimum and economical in comparison with inoculum dosage of 5 mL because not much of difference in COD removal is seen between them. Thus the optimized growth parameters for fungal consortium are 4% of inoculum concentration at 30°C for 6 d in natural pH of 4.02.

3.3.3. Comparative analysis of treatment efficiencies by bacterial and fungal cultures

Maximum cyanide and COD removal efficiencies for mixed bacterial culture were 41.12% and 53.07% respectively which were low because of the low pH of wastewater as optimum pH for bacteria is usually in between pH 6.5–8. Maximum cyanide and COD removal efficiencies for fungal consortium were 63.77% and 74.3% respectively which were higher than mixed bacterial culture because fungus can grow better low pH of wastewater also, the fungus is known to tolerate a toxic environment of cassava wastewater more efficiently than mixed bacterial culture. The removal efficiencies are higher in the fungal treatment than mixed bacterial culture; thus fungi can grow better in acidic pH which is in correlation with previous reports [20]. *Aspergillus terreus, Fusarium oxysporum, and Neurospora crassa.* The results also reveal that fungal consortium is highly compatible to the harsh condition of the wastewater and able to utilize the starch from it more efficiently, which is in correlation with the previous reports wherein fungus treatment gave better COD removal in paper and pulp wastewater [26]. Also, it was substantiated from recent studies that *Aspergillus niger* was better in breaking down starch from cassava waste than Bacillus sp. [27].

3.4. Comparative analysis of the treatment efficiencies of free, immobilized and biofilm culture for cassava wastewater treatment

3.4.1. Treatment of wastewater using immobilized fungal consortium in calcium alginate beads

The immobilized fungal consortium was used to treat the cassava wastewater, and under the optimized conditions, the removal efficiency reached a maximum value of 76.5%. The decrease in COD removal can be supported by the fact that *T. harzianum* and *T. viride* are macrofungi and mycelium growth of such fungi is difficult in entrapped Ca Alginate beads. For effluent treatment, it is important that the fungus develop filamentous strands. Furthermore, a similar kind of study that showed that COD removal efficiency in an immobilized system using *Aspergillus sp.* in dairy wastewater was low [28].

3.4.2. Treatment of wastewater using fungal consortium as a biofilm

The fungal consortium was used as a biofilm, and the maximum COD removal efficiency was 88% within 6 d of the treatment process. The result can be justified by the fact that plastic support material provides a large surface area for mycelium growth of *T. harzianum* and *T. viride* due to which more growth of fungal consortium is observed, and thus more amount of starch was utilized by fungi which results in higher COD removal from cassava wastewater. Higher COD removal shows that biofilm batch reactor is efficient enough to treat cassava wastewater. From Fig. 5, it can be seen that cyanide removal efficiency was seen highest in biofilm batch reactor with 98.19% and then 87% in the immobilized batch reactor. The cyanide removal efficiency is seen as a minimum in suspension with only 77%. The results are by many other authors. This may be attributed by the fact that plastic support material and Ca Alginate beads provided higher resistance to the toxic environment and thus fungus can thrive such harsh environment and able to secrete the enzymes like cyanide hydratase for degradation for the cyanide from the cassava wastewater [29].

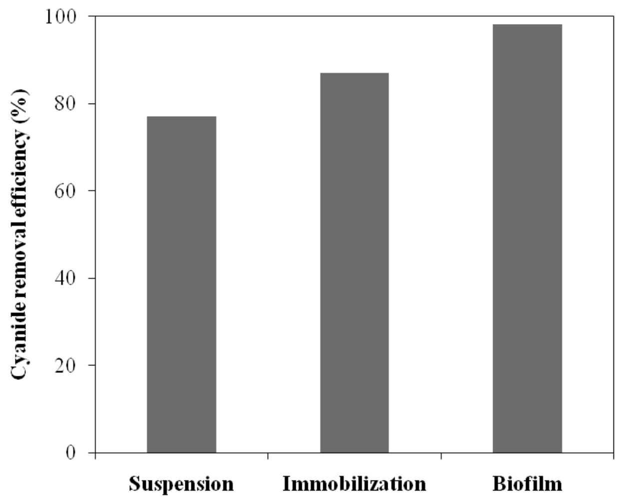

Fig. 5. Comparison of cyanide removal efficiency using fungal consortium.

The maximum COD removal efficiency using mixed fungal consortium under the optimized condition for 6 d in suspension, immobilized and Biofilm batch reactor is 83.5%, 76.5%, and 88% respectively and illustrated in Fig. 5. Mixed fungal consortium immobilized in Ca-Alginate beads showed less efficient COD removal than mixed fungal consortium in suspension showing that treatment in suspension is a better option for treating the cassava wastewater with high starch. Furthermore, comparison of mixed fungal consortium in the biofilm reactor with suspension shows that the biofilm reactor gives higher COD removal. Thus, the results obtained indicate that the use of biofilm batch reactor is a most effective method for treatment of cassava wastewater containing high starch content and low pH which is in correlation with the previous studies [30]. It was concluded that a significant increase in the degradation of lignin and the removal of COD were seen using the attached growth of *Phanerochaetechrysosporium*, *Pleurotusostreatus*, *Lentinusedodes* and *Trametesversicolor* in paper and pulp wastewater.

The final characterization of the treated effluent by free, immobilized and biofilm was done. It is seen that the decrease in other parameters like TS, TDS, TSS and BOD is observed as shown in Table 1. The pH of free, immobilized and biofilm of fungal consortium after six days treatment is 8.38, 8.44 and 8.53 respectively. The increase in pH is seen in all three situations from initial pH of 4.02. The reason behind the increase may be due to the production of ammonia during enzymatic degradation of cyanide by the fungal consortium. Also, it has been reported by many authors that during fungal growth no acidification occurs, and fungus can utilize the acid from the wastewater. Hence, even if present there are no chances of pH reduction in the system. Increase in pH after treatment period is also in correlation to the statement that fungi can alter the pH of the medium due to the differentiated transport of cation and anions during substrate transportation.

4. Conclusion

Eleven colonies were isolated from the cassava wastewater, and four colonies were successfully screened. Maximum cyanide and COD removal efficiencies for mixed bacterial culture were 41.12% and 53.07% respectively which were low due to the low pH of wastewater as compared to the optimum pH of 6.5–8 for bacteria. Maximum cyanide and COD removal efficiencies for fungal consortium were 63.77% and 74.3% respectively which were higher than mixed bacterial culture because fungus can grow better low pH of wastewater. Moreover, the fungus is known to tolerate a toxic environment of wastewater much more efficiently than mixed bacterial culture. Treatment by mixed fungal consortium also resulted in a higher increase of pH, i.e., 8.14 from 4.02 thus reducing the need for buffers after treatment. As fungal consortium showed higher removal efficiencies than mixed bacterial isolates, it indicates that fungal treatment can be a promising and efficient technology to treat cassava wastewater. It was also substantiated that the rate of bioremediation process is proportional to the amount of microbial biomass. Hence, six days of treatment using fungal consortium which showed maximum biomass of 3.721 g/L as dry weight can be considered as active treatment period for fungal treatment. Moreover, it was also observed that fungal immobilization in Calcium-alginate beads has better cyanide removal efficiency (87%) than in suspension method (77%) while it is the least effective method for COD removal (76.5%). The biofilm batch reactor with highest cyanide and COD removal efficiency of 98% and 88% respectively can thus be considered as the best method amongst the other two for treatment of cassava wastewater.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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