STUDY OF EXTRACELLULAR BIOFLOCCULANT PRODUCTION FOR WASTEWATER TREATMENT

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Abstract: Industrial process is a potential source of pollution and requires a specific treatment for the waste water produced. Flocculation is the most widely used process for treating the wastewater. The use of bioflocculant has attracted huge scientific interest because of their biodegradability and safety for ecosystems. In the present study twenty four bioflocculant producing bacteria were isolated from various industrial wastewaters, sludge samples, river water and soil on basal bioflocculant screening agar. During preliminary screening nine isolates were identified as effective bioflocculant producing micro-organisms, as they yielded more than 70% flocculation activity. The isolates SF 17 and TS 5 and their consortium were studied further for flocculant production. Various parameters like carbon source, nitrogen source, pH, temperature and inoculum dosage were studied to obtain the maximum flocculant production. Among the different carbon sources studied, sodium carbonate was the most favorable carbon source supporting a flocculating activity of 75%, 71% and 81% for SF 17, TS 5 and consortium respectively. Among the various nitrogen sources examined, the mixture [Urea+ Yeast extract+ $(NH_4)_2SO_4$] proved to be the best nitrogen source with the highest flocculating activity of 68% 75% and 93% respectively. The optimum pH was found to be 7.0 for bioflocculant production. Also the bioflocculant was found to be thermostable at the temperature range of 37-100°C. An inoculum size of 2% (v/v) supported an optimum flocculating activity of 83%, 90% and 96% respectively.

I. INTRODUCTION

Flocculation is a process whereby colloids come out of suspension in the form of flocs and is most commonly used in wastewater treatment. It is mainly achieved with the aid of flocculants which quickens the process (Kurane *et al.,* 1986). Flocculants, act on the molecular level on the surface of the particles thereby reducing the repulsive forces and mounting attractive forces. Their addition enables dispersed particles to aggregate together to form flocs of a size and allows them to settle speedily for clearing the system. Over the last decade, a variety of flocculants comprising of inorganic, organic and natural bioflocculants have found widespread applications in several industrial and waste water treatment processes such as pharmaceutical, fermentation, food industries dredging and downstream processing. Microbes, especially bacteria have shorter generation times, are versatile and can produce extra cellular polymeric material which can flocculate; the latter and those which are obtained from natural sources have been termed as **'bioflocculants'**. These are exemplified by chitosan, gelatin, starch, cellulose and sodium alginate. These naturally occurring flocculants from renewable biomass are safe and biodegradable, cheap and non-toxic, but show weak activity in application (Kurane *et al.,* 1986; He *et al.,* 2004; Abdel- Aziz *et al.,* 2011).

Several bioflocculants from different microorganisms have been reported recently. Flocculants produced by *Rhodococcus erythropolis S-1* (Kurane, et. al.1986) are predominantly protein in nature, whereas, those produced by *Alcaligenes* sp B-18 and B*acillus sp*. DP-152 (Suh, *et. al*., 1997) are polysaccharide in nature. On the other hand, the flocculants produced by *Arcuadendron sp*.TS-4 were shown to be a glycoprotein. *Vergibacillus* sp. produced bioflocculant having 87% uronic acid (Cosa *et al.,* 2013). The bioflocculants produced by *Rhodococcus erythropolis* are reported to be glycolipid and protein (Kurane *et al.,* 1994b; Koizumi *et al.,* 1991). The biodegradability of microbial flocculants have initiated research into screening, characterization and structural identification of polymeric flocculants elaborated by the microbes (bacteria, fungi and algae), worldwide.

The present study aimed at finding a consortium of micro- organisms capable of producing bioflocculants with high efficiency. The study aimed at optimizing the physical and chemical factors to enhance the production of bioflocculants. An in-situ analysis of the efficiency of the bioflocculant in the removal of suspended particles from the sewage samples was also analysed.

II. MATERIALS AND METHODS

2.1 Enrichment and Isolation of Bioflocculant producing bacteria

Six wastewater samples Kalyan Dombivili Municipal Corporation region were collected from different sources and one garage soil sample were collected in screw cap sterilized bottles and analyzed within 4 hours from collection. 0.1 ml of appropriate samples was plated on to Nutrient agar plates. All plates were incubated at 37°C for 24 - 48 hours. A screening experiment was first conducted on all the isolated strains to select the most potent organisms in bioflocculant production. The Basal Bioflocculation Screening medium (BBSM) known to enable bacteria to produce bioflocculants was used.

A loopful of each bacterial colony were inoculated separately into 50 mL of the BBSM broth, incubated with shaking at 160 rpm for 72 h at 28°C. At the end of incubation, cultured cells were centrifuged at 4,000 rpm for 30 min. The cell-free supernatant was used to determine flocculant activity by the method described by Kurane *et al.* (1994) where, kaolin clay was used as the suspended solid using spectrophotometric method. The strain with the highest flocculating activity was selected for further investigations. All assays including the control were performed in triplicates and the flocculation activity was calculated.

2.2 Optimization of the bioflocculant production

The Isolates showing biofloculation capacity were selected for further optimization studies to determine the carbon and Nitrogen source, pH, inoculum size and thermal stability. The following carbon sources each: Lactose, maltose, fructose, sucrose and sodium carbonate were used for carbon substrate and for nitrogen source, Urea, Yeast Extract, Ammonium Sulphate, Ammonium Chloride and Mixture [Urea+ Yeast extract+ $(NH_4)_2SO_4$]. The effect of pH was studied for the pH 3.0, 5.0, 7.0, 9.0 and 10.0. Different concentrations of the pre-inoculum were inoculated in production medium yielding bioflocculant solutions concentrations of 1-5% (v/v). The medium was incubated with shaking (120 rpm) at 28°C for 72 hours. The flocculation assay of the different bioflocculant solutions was performed. The thermal stability of the bioflocculant was assessed by incubating solutions of the bioflocculant at 37°C, 55°C, 70°C, 90°C and 100°C for 30 mins after which residual flocculating activity was determined. Bioflocculant purification was achieved according to the procedure described by Okaiyeto *et al.* (2013).

The domestic wastewater was collected from local residential area. The textile effluent wastewater was collected from a textile factory for the experiment. Some physicochemical parameters were measured in collected water samples. These parameters including; turbidity, biological oxygen demand (BOD), Chemical oxygen demand (COD), pH of the domestic water and textile wastewater, OD at 550nm. All physicochemical parameters were measured according to standard methods for the examination of water and wastewaters.

2.3 Wastewater treatment using bioflocculant

For treating wastewaters, the different bioflocculants were added into 500 mL wastewater and incubated for 5 days. After 5 days, the supernatant was taken to analyze flocculating effect. For flocculation efficiency assays, the wastewaters were used instead of a kaolin suspension. 100 mL wastewater sample was taken in a 250 mL beaker where 10 mg of the lyophilized product of bioflocculant was added and kept for stirring on magnetic stirrer initially at 200 rpm for 10 min followed by 40 rpm for 5 min and then allowed to settle for 10-20 min. The supernatant was carefully removed and used for analysis. In addition, the wastewater was subjected to stirring without exposure to the bioflocculant as a control. The residual BOD, COD, pH, OD at 550 nm and turbidity were determined after the bioflocculant treatment and the removal efficiency was calculated.

2.4 Dye Removal By The Bacterial Flocculants

The two dye solutions were used in this study Eriochrome Black T (50 mg L^{-1}) and new Fuchsin (100 mg L^{-1}). In decolorization experiments, 0.3mL culture broth and 0.3mL $CaCl_2$ (1wt %) were added to 10mL dye solutions, then the pH of the suspension was adjusted to 8 with NaOH solutions (10 wt %). The mixture stirred for 1 min, held for 10 min, and the supernatant was taken for analysis. The absorbance of each sample was measured using spectrophotometer at the maximum wavelength of each dye (543 and 598 nm for new Fuchsin, Eriochrome Black T, respectively). The residual concentration of the dye in the samples was then calculated, and the decolorization efficiency was calculated based on the initial dye and final dye concentrations after treatment.

2.5 Heavy Metal Removal Efficiency of Bacterial Bioflocculants

The efficiency by which bacterial bioflocculants removed heavy metal was determined using the modified method using heavy metals without kaolin clay. The metal salts used were copper sulphate, nickel nitrate and manganese chloride. Various concentrations of heavy metal solutions ranging from 10 to 10,000 ppm were prepared. 0.3 mL of bioflocculant solution was put into test tube containing 10 ml of each appropriate metal-salt solutions and shaken at 100 xg for 24 hours at 30°C. After 24 hours, the supernatant was taken for analysis. The quantity of metal removed from the solution, i.e. bound to the polymer, was calculated by measuring the flocculant activity as described above. The percentage of removal of each metal was calculated.

III. RESULTS AND DISCUSSION

3.1 Sampling

Six different sludge samples of wastewater treatment plants, textile industry, domestic sewage water, river water samples and soil samples were collected from different locations in sterile bottles. The samples were collected from the following sites (Fig 1).

Fig 1: Sites of sample collection

For the screening of flocculant producing bacteria, a total of 24 bacteria were isolated from enriched sludge samples of different wastewater treatment plant, domestic sewage, textile industry, river water samples and garage soil samples after 24-48 hours of incubation.

3.2 **Flocculation Activity**

Based on the highest flocculating activity, five isolates were selected and coded as US 1, TS 5, SF 14, SF 17 and GS 22. The best two isolates TS 5 and SF 17 that showed the highest flocculating activity of 78% and 86% respectively for Kaolin clay suspension (5 g/L) were selected for further studies (Table 1).

Table 1: Flocculation Efficiency of various isolates

Isolates	Flocculation Efficiency (%)	Isolates	Flocculation Efficiency (%)
US 1	72.72	SC 13	62.12
US 2	69.69	SF 14	71.21
US 3	45.45	SF 15	48.48
TS 4	54.54	SF 16	52.34
TS 5	78.86	SF 17	86.36
TS 6	68.18	DW 18	64.69
SW 7	66.66	DW 19	51.51
SW 8	69.69	DW 20	69.69
SW 9	51.51	DW 21	66.66
SC 10	37.87	GS 22	72.72
SC 11	31.81	GS 23	68.18
SC 12	59.09	GS 24	52.34

3.3 Optimization of the bioflocculant production

After 72 hrs of fermentation, optimal bioflocculant was released into the culture broth as indicated by flocculating activity (65%) was observed at 72 h of cultivation beyond which flocculating activity started to decline . A decline in cell growth and flocculating activity at late growth phase may be attributed to the depletion of nutrients in the production medium as well as to the production and release of bioflocculant-degrading enzymes which utilize the produced bioflocculant as a carbon source (Li et al., 2009; Zaki et al., 2011). Similarly, a bioflocculant produced by *Serratia ficaria* reached its maximum flocculating activity in the early stationary phase (72 h) before an observed a slow decrease after 84 h which was attributed to autolysis and enzymatic activity (Gong et al., 2008). These findings are an indication that the production of the bioflocculant is associated with cell growth rather than with cell autolysis (Lu et al., 2005; Gao et al., 2006). The majority of reported studies in the literature indicate that bioflocculants are produced during active growth phase of microorganisms (Salehizadeh and Yan, 2014; Lian et al., 2008; Prasertsan et al., 2006; Nwodo et al., 2013). The present study seems to show a similar phenomenon.

3.3.1 Effect of Carbon Sources on Bioflocculant Production

Carbon source play an important role in enhancing bioflocculant production and different microorganisms prefer different carbon sources for bioflocculant production (Piyo *et al*., 2011). The effects of various carbon sources on bioflocculant production were investigated (Fig.2). Among the different carbon sources studied, sodium carbonate was the most favorable carbon source supporting a flocculating activity of 75%, 71% and 81% for SF 17, TS 5 and consortium respectively and sucrose being the least favorable carbon source with flocculating activity of 42%, 61% and 70%respectively. Sodium carbonate was then chosen as the sole carbon source for all subsequent experiments.

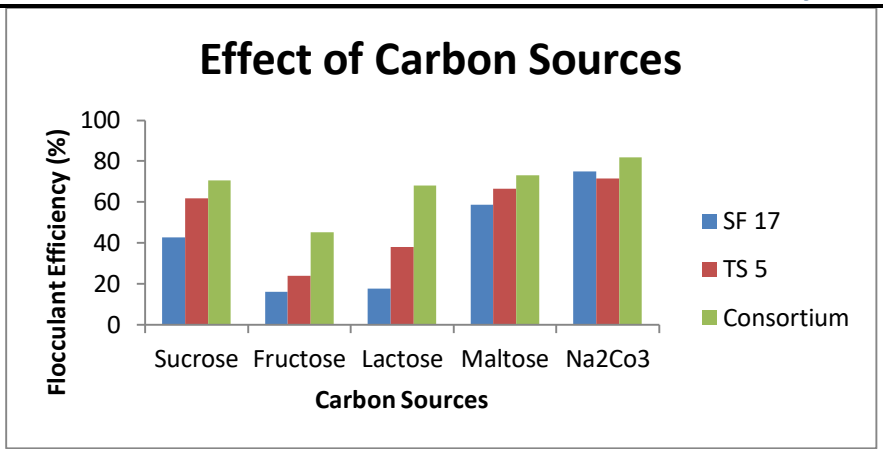

Fig2: Effect of carbon sources on bioflocculant production

Among the carbon sources studied, monosaccharides *i.e.*, glucose and galactose and disaccharides *i.e.*, sucrose and lactose supported bioflocculant production at 24 h with 87%. Flocculating activity, whereas starch was slowly utilized and took 72 h to achieve same flocculating activity. Xiong *et al*. (2010) reported the same results for *B. licheniformis*.

3.3.2 Effect of Nitrogen Sources on Bioflocculant Production

Various microorganisms require the presence of either organic or inorganic nitrogen sources for bioflocculant production. The effect of organic (yeast extract, urea) and inorganic nitrogen sources (ammonium sulphate, ammonium chloride) and the combination of yeast extract, urea and ammonium chloride on bioflocculant production was investigated. As shown in (Fig.3), among the various nitrogen sources examined the mixture proved to be the best nitrogen source with the highest flocculating activity of 68% 75% and 93% respectively. This experiment suggested that a single nitrogen source is not sufficient for the production of bioflocculant.

However, ammonium sulphate was the best inorganic nitrogen source as compared to other inorganic sources for the growth of *Klebsiella pneumonia*e NY1 and its flocculating activity. *Bacillus clausii* required peptone and yeast extract giving 88% flocculation activity which is at par with our study (Adebayo-Tayo and Adebami, 2014).. Most of the bacteria required a mixture of yeast extract, urea and ammonium sulphate e.g: *Rhizobium radiobacter* and *Bacillus sphaericus* (Zhao *et al*., 2012).

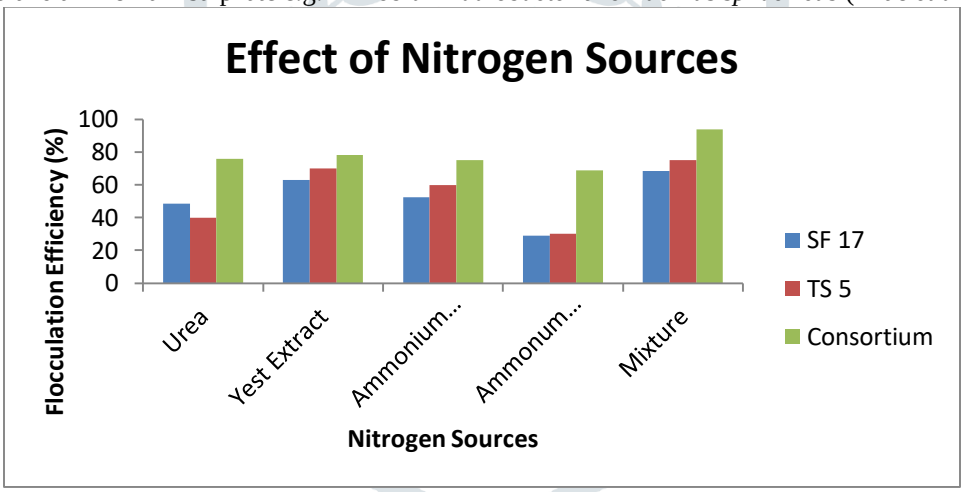

Fig.3: Effect of nitrogen sources on bioflocculant production

3.3.3 Effect of initial pH On Bioflocculant Production

Initial pH for bioflocculant production varies with different microorganisms (Salehizadeh and Yan, 2014). The effect of initial pH of the culture medium on bioflocculant production was investigated at a pH range of 3-10 and the results are depicted in (Fig.4). The results depicted that maximum production was observed at pH 7, which was optimum for bioflocculant production after which there was a decline in flocculant activity. The decline in activity with increasing pH might be due to alkaline degradation of polysaccharide, leading to reorganization of its remains or disintegration of the polysaccharide chain.

Similarly, according to He *et al*. (2010), the flocculating activity of a bioflocculant produced by *Halomonas* sp. V3a' was above 80% in the pH range of 3 to 11 and the highest flocculating activity of 97% was recorded at pH 7.

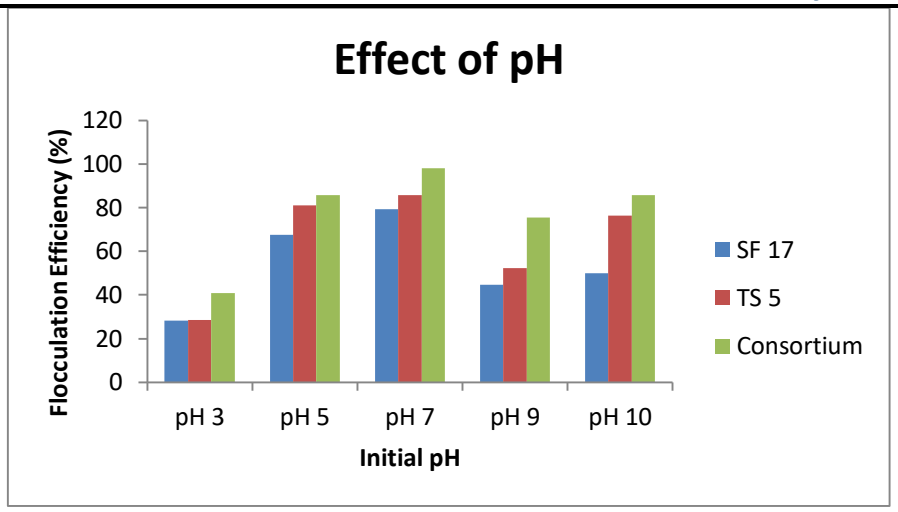

Fig.4Effect of initial pH on bioflocculant production

3.3.4 Effect of Temperature Stability On Bioflocculant Production:

The effect of heat on the bioflocculant activity was investigated over a heating period of 30 mins at a temperature range of 37-100°C. Optimum flocculating activity above 50% was recorded over this temperature range thus indicating relative thermo stability characteristics of the bioflocculant (Fig.5). Similar findings have been reported by Gao *et al*., (2006) for a bioflocculant produced by *Vagococcus* sp. retained its stability when heated at 100°C with the residual flocculating activity of culture broth and purified flocculant being 86.5% and 87.2%, respectively.

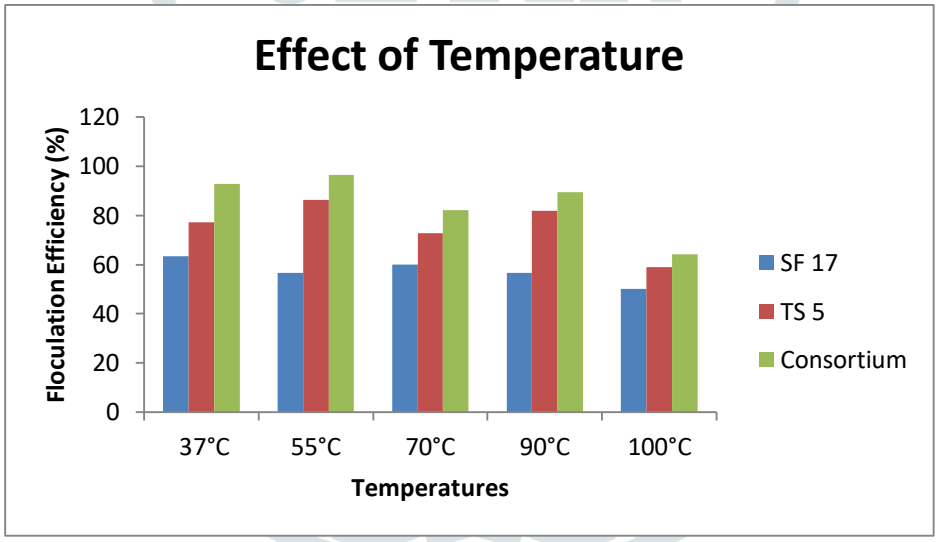

Fig.5 Effect of temperature stability on bioflocculant production

3.3.5 Effect of Inoculum Size on Bioflocculant Production

Inoculum size is of great significance factor in bioflocculant production and cell growth (Salehizadeh and Yan, 2014). Salehizadeh and Shajoasadati (2001) reported that a small size of inoculum can prolong the lag phase, while the large inoculum size will create niches of the strain to overlap excessively, thus inhibiting bioflocculant production. The effect of inoculum size on bioflocculant production was investigated as shown in (Fig. 6). An inoculum size of 2% (v/v) supported an optimum flocculating activity of 83%, 90% and 96% respectively, beyond which a decrease in activity was observed, thus making 2% and inoculum size of choice for all subsequent culture experiments. Similarly, Zhang *et al*., 2007carried out studies on bioflocculant production by the following bacterial strains, *Serratia ficaria* (Gong *et al*., 2008), *Klebsiella pneumoniae* YZ-6 (Luo *et al*., 2014) and by multiple-microorganism consortia of *Staphylococcus* sp. and *Pseudomonas* sp. showed maximum flocculating activity being attained at 2% inoculum size.

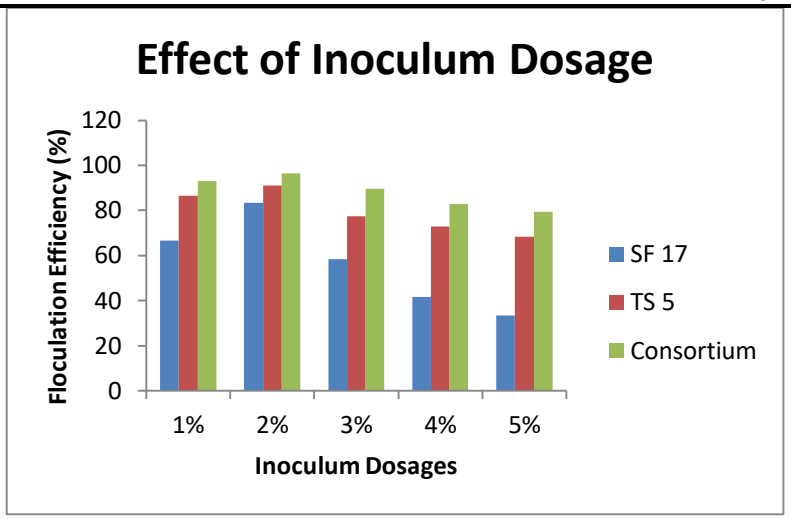

Fig. 6: Effect of inoculum size on bioflocculant production

The extracted dried flocculant looked like white powder (Fig 5.8). The yield of the bioflocculant obtained after partial purification by chloroform and n- butylalcohol (5:2) treatment and simultaneous ethanol precipitation was higher in case of consortium as compared to individual isolates. The yield of the bioflocculant from the consortium was 0.62g/L of culture broth which was acceptable for bioflocculant production. It was similar to the previous report that about 1.58–2.19 g bioflocculant could be obtained per 1000 mL fermentation liquid by Lian *et al.* (2008).The yield of bioflocculant from individual isolates SF17 and TS 5 was 0.51 g/l and 0.45g/l respectively. Hence the bioflocculant yield recovered from the consortium was quite comparable with that produced from the pure individual cultures. The yield of bioflocculant is also an important factor to consider with respect to its industrial application. Our bioflocculant was 0.62 g/L and hence may be good for application.

3.4 Wastewater Treatment by Bacterial Flocculants

The process of flocculation is a critical physicochemical treatment step in the treatment of industrial wastewater to reduce both the suspended colloidal substances responsible for wastewater turbidity and organic matter that contributes to the BOD and COD content of the water. The utilization of coagulants/flocculants in water and wastewater treatment may enable destabilization of particulate matters, resulting in the formation of flocs and, consequently, improved sedimentation. However, this may vary with the quality of water/wastewater being treated. The bioflocculant produced by the consortium culture of SF 17 and *TS 5* exhibited high flocculating efficiency for kaolin suspension, hence it was subsequently tested on wastewaters. The jar test experiment on the treatment of textile effluent and domestic wastewater was performed with the test bioflocculant and the initial pH, O.D., turbidity, COD, BOD of the textile and domestic wastewater are shown in the [Table 2].

Table2: Physico-chemical characteristics of raw effluent and wastewater

Parameters	Domestic Wastewater	Textile Effluent
O.D. at 550 nm	0.32	0.26
pH	7.0	6.5
Turbidity (%)	0.53	0.23
COD (mg/L)	551	915
BOD (mg/L)	374	690

The treatment of industrial effluent and domestic wastewater with bioflocculants at 100mg/L concentration exhibited fast and efficient flocculation with a concomitant reduction in O.D., pH, turbidity, BOD and COD of textile and domestic wastewaters. it was observed that consortium was proved a better bioflocculant as compared to individual isolates because a notable reduction in pH, O.D., turbidity, COD and BOD was observed from both the wastewater samples. The flocculation of wastewaters by the purified bioflocculant revealed that the highest bioflocculant flocculant efficiency of domestic wastewater and textile wastewater were observed by consortium at 76% and 86% respectively. There are many reports available on reduction in COD and turbidity with the use of bioflocculants. According to Feng *et al.* (2009), bioflocculant of *Klebsiella* sp. exhibited excellent turbidity removal (99%) and COD reduction (68.4%) at lower concentration (40 mg/L).

3.5 Dye Removal Efficiency of Bioflocculant

The New Fuchsin, Eriochrome Black T were used to measure decolorization of bioflocculant. Until now there have been few reports about treating the two dyes. The decolorization efficiency for new fuchsin was 66%, 61% and 64% after the treatment by SF 17, TS 5 and Consortium respectively, while the decolorization efficiency for Eriochrome Black T was 71%, 61% and 68% after the treatment by SF 17, TS 5 and Consortium respectively. It was found that SF 17 had strong decolorizing ability as compared to other bioflocculants (Fig. 7).

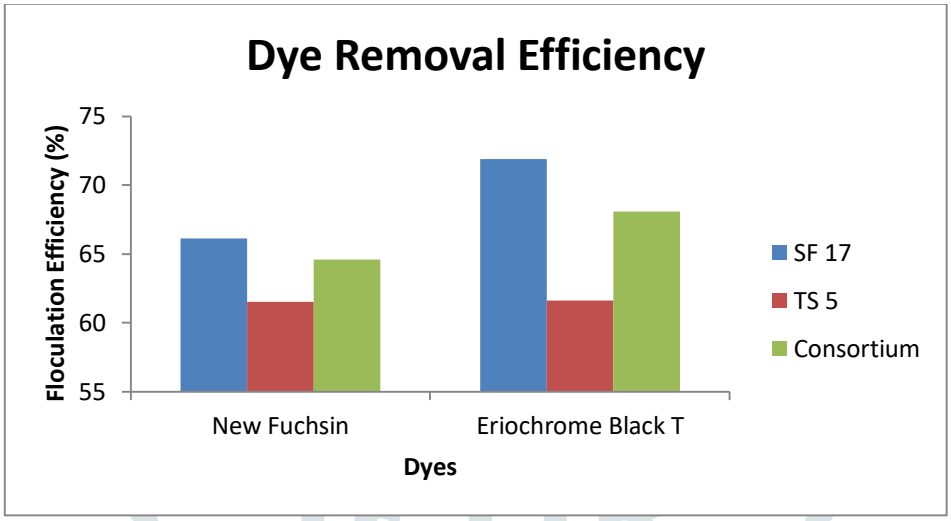

Fig. 7: Dye removal Efficiency of Bioflocculant

Recently, Zhang *et al.* (2010) reported that bioflocculants produced by bacterial strains xn11 + xn7 were effective in decolorizing basic fuchsin (100 mg L−1) but less effective in decolorizing reactive black (50 mg L−1), exhibiting a decolorization efficiency of 93 and 35%, respectively.

3.6 Heavy Metal Removal Efficiency of bioflocculant

The treatment with bioflocculant also helped in metal removal from different heavy metal solutions. As shown in (Fig.8), the consortium bioflocculant exhibited highest removal efficiency of 77.77% removal of Nickel nitrate 86.76% removal of Copper sulphate and 73.33% removal of manganese chloride. The other bioflocculants SF 17 and TS 5 revealed removal efficiency of 68% and 59% removal of Nickel nitrate, 79% and 50% removal of copper sulphate and 68% and 60% removal of manganese chloride respectively. Thus, flocculation also helps in removal of different types of heavy metals along with various other applications. A bioflocculant produced by *Bacillus firmus* resulted in the removal of lead (98.3%), copper (74.9%) and zinc (61.8%) from aqueous solution at optimum pH values of 4.5, 4 and 6, respectively (Salehizadeh and Shojaosadati, 2003).

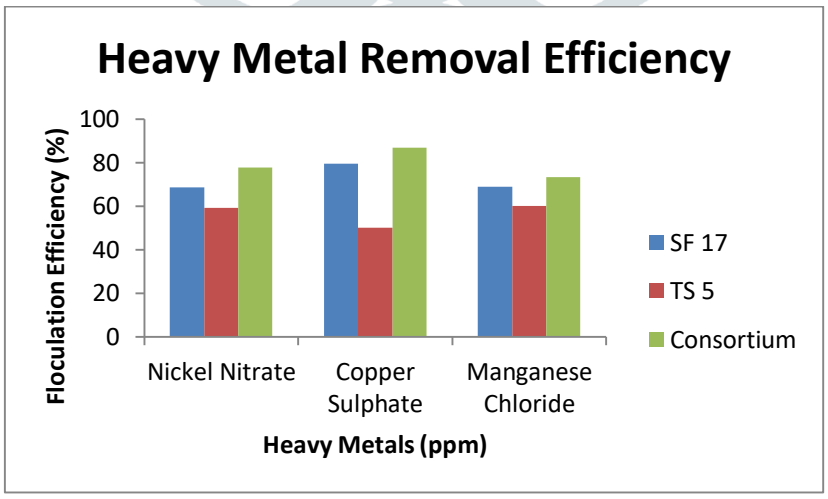

Fig.8: Heavy metal removal efficiency of bioflocculants

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