STUDIES ON POLYMERIC BIOFLOCCULANT PRODUCING MICROORGANISMS

A

Project submitted

In partial fulfillment for the award of

Degree of Master of Science in Biotechnology

BY
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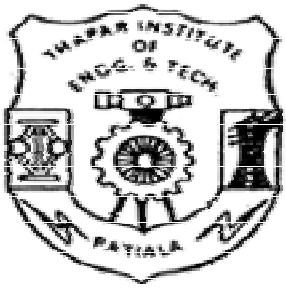

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on Polymeric Bioflocculant producing Microorganisms" by Pankaj Lachhwani** in partial fulfillment of the requirements for the award of the degree of Masters of Sciences in Biotechnology, to Thapar Institute of Engineering and Technology (Deemed University), Patiala is a record of student's own work carried out by him under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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ABSTRACT

Industrial waste water, sludge and soil samples were screened for bacterial isolates capable of producing flocculants. Out of the fourteen isolates obtained, two isolates possessed significant flocculating abilities and were used for further studies. The effects of cultural conditions viz: pH, carbon, nitrogen sources, temperature and starvation were investigated on the production of bioflocculants by theses two isolates. Isolate 1 was found to prefer glucose, ammonium sulfate and peptone for flocculant production; the optimum pH for flocculant production were 7.5. Na^{+} and Mg^{++}, static conditions and carbon starvation decreased flocculant production. Isolate 2 produced lower amounts of flocculant as Compared with isolate 1. when this cultured with glucose, ammonium sulfate and peptone. The optimum pH for flocculant production were also 7.5 respectively.. Na^{+} and Mg^{++}, Static conditions and carbon starvation decrease flocculant production marginally.

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In the end, I am thankful to the Almighty for blessing me to complete this work successfully.

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CONTENTS

INTRODUCTION

Flocculants are substances having a synthetic or natural origin that are used as the sedimentation aids to bring about the solid liquid separations by the process of flocculation in industrial plants. Flocculants are either cationic or anionic charged and are available in wide range of the molecular weights. The use of the flocculants is necessary because of suspended solids in the aqueous solution exhibit Brownian movement which keep them in constant motion and inhibit settling; the purpose of the flocculant is to neutralize the like charges in suspension by coagulating and flocculating them into large size. The larger is the particle size faster is the settling rate, hence improved the settling and cleaner the supernatant are achieved rapidly. Owing to presence of negative surface charges on this particle, the electrostatic repulsion overwhelms the Van der-waals attractive forces, preventing aggregation, by adding positively charged flocculants that neutralizes the negatively charged particles allowing the particles to colloid aggregate as floc.

Over the last decade, a variety of flocculants comprising of inorganic (Polyaluminium chloride, Aluminum sulfate) organic (polyacrylamide, polyethylene amine) and natural bioflocculants (gelatin, chitosan, guargum and microbial flocculants) have found widespread applications in several industrial and waste water treatment processes such as pharmaceutical, fermentation, food industries dredging and downstream processing among the different types of flocculants in use, chemical or synthetic flocculants has higher popularity , this is attributed to several properties of these flocculants viz: effective flocculating activity, low cost and versatile tailor ability (Saliehizadeh, et. al., 2002).

Despite the singular advantages that synthetic or chemical flocculants provide, their status has not been favorable currently and there have been concerns on their safety. Most of high molecular weight are recalcitrant. Monomers of polyacralamide are potent carcinogen and neurotoxic to humans and other animals (Vanhoric, et. a., 1983). They have detrimental effect both on flora and fauna. Aluminium has been shown to cause Alzheimer's disease. The huge amounts of flocculants used everyday globally and particularly in India is a cause

of concern, considering the health problems created by the flocculants. Naturally occurring polymeric materials have been shown to cause flocculation, such materials have been derived mostly from plants; however a cheap and easy alternative, which is currently gaining popularity, is microbes. 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'**. Since bioflocculation is a dynamic process resulting from synthesis of extra cellular polymers by living cells, soil and activated sludge are considered as the best source for screening and isolation of such flocculant producing bacteria.

Bioflocculants possess several advantages: they are convenient to use, need to be added in lesser quantity (1-5 ppm) and form strong and larger flocs without affecting the pH of the working medium allowing better settling than those of the simple coagulating electrolytes. They are also shear stable to a large extent and importantly, biodegradable; additionally, they can be tailored. Structurally, they are variable; some examples illustrate this point and highlight the diversity of microbial flocculants: Several bioflocculants from different microorganisms have been reported recently. Flocculants produced by *Rhodoocus 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. The uniqueness and biodegradability of microbial flocculants have prompted research into screening, characterization and structural identification of polymeric flocculants elaborated by the microbes (bacteria, fungi and algae), globally. In India, however, there is a paucity of information on the above aspects and further research is needed in order to develop better and environmentally safer alternatives to the synthetic flocculants.

Scope of the dissertation

The objectives of this work was to screen bacterial isolates capable of producing flocculants, optimize the cultural conditions for flocculant production and study some of the physico-chemical properties of these flocculants.

REVIEW OF LITERATURE

Flocculation is an essential phenomenon used in domestic and industrial wastewater treatment for separation of suspended solids from wastewater. Flocculation is achieved with the help of flocculants, which are the natural or synthetic substances that facilitate the agglomeration or aggregation of the coagulated particles to form floccules and there by hasten the gravitational settling of suspended solids in solution. These small flocs can be build up into larger aggregates by flocculation, with the larger particles formed in this way giving higher rate of sedimentation. Flocculants have the wider range of the applications, -in mineral industry (for ore leaching treatment after floatation, treatment of tailings to prevent pollution), in industrial waste treatment (Blast furnace washing), Surface treatment industry, Petroleum refinery effluents), sewage and municipal waste (In the physicochemical reactions, Prior sludge dewatering), chemical industry (water reuse at the clarification stages of the phosphoric acid, di-calcium phosphate brine electrolyte and magnesia production, Paper industry (Retention of fines and fibers), Drainage improvement, Raw water treatment, Potable water treatment, Secondary oil recovery and other industrial purposes like Oil recovery , and Dredging. It has also significant uses in the food and fermentation industry (Gutcho, et. al., 1977).

Mechanisms

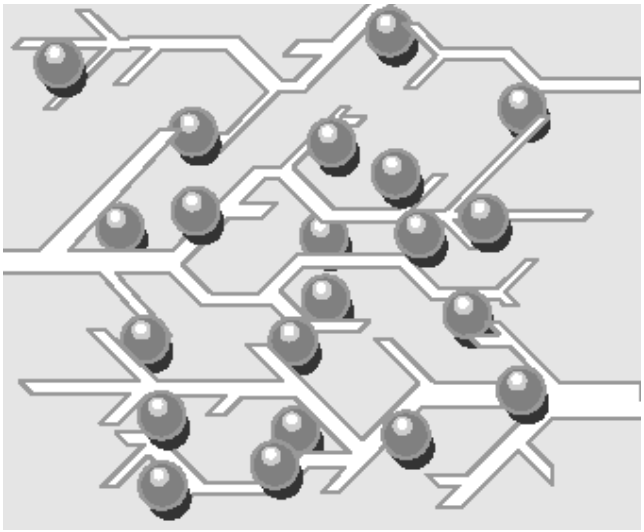
A significant amount of research has been performed to better understand the mechanisms of bioflocculation and solid/liquid separation processes. There are two types of mechanisms given bellow:

1) Bridging

Bridging occurs when a flocculant forms threads or fibers which attach to several colloids, capturing and binding them together. Inorganic primary coagulants and organic polyelectrolytes both have the capability of bridging. Higher molecular weights mean longer molecules and more effective bridging. Bridging is often used in conjunction with charge neutralization to grow fast settling and/or shear resistant flocs. For instance, alum or a low molecular weight cationic polymer is first added under rapid mixing conditions to lower the charge and allow microflocs

to form. Then a slight amount of high molecular weight polymer, often an anionic, can be added to bridge between the microflocs. The fact that the bridging polymer is negatively charged is not significant because the small colloids have already been captured as microflocs.

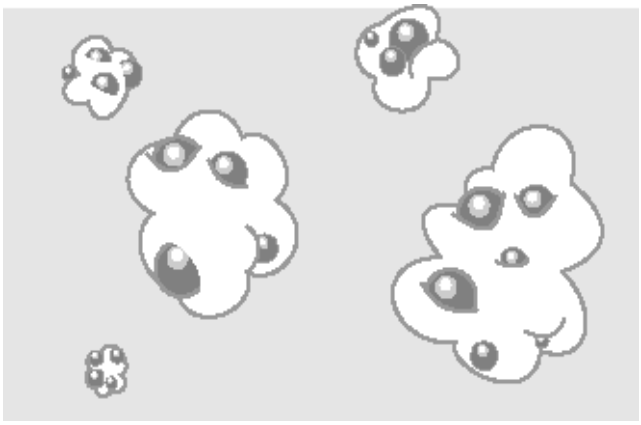
Each polymer chain attaches to many colloids.



2) Colloid Entrapment

Colloid entrapment involves adding relatively large doses of flocculants, usually aluminum or iron salts which precipitate as hydrous metal oxides. The amount of flocculant used is far in excess of the amount needed to neutralize the charge on the colloid. Some charge neutralization may occur but most of the colloids are literally swept from the bulk of the water by becoming enmeshed in the settling hydrous oxide floc. This mechanism is often called **sweep floc.**

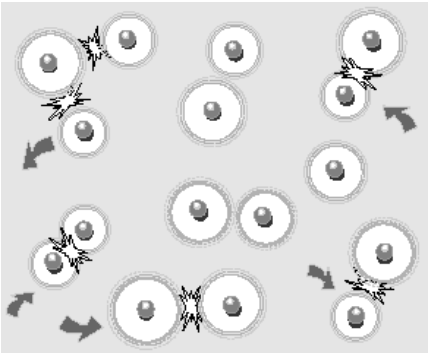
Colloids become enmeshed in the growing precipitate.



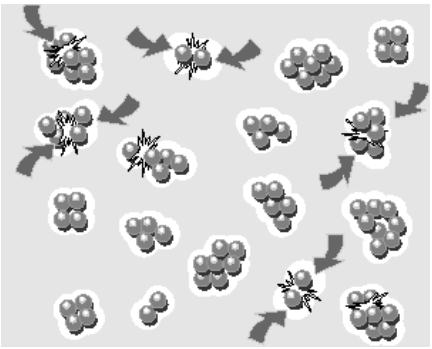
Particle Charge Prevents flocculation

The key to effective flocculation is an understanding of how individual colloids interact with each other. Turbidity particles range from about 0.01 to 100 microns in size. The larger fraction is relatively easy to settle or filter. The smaller, colloidal fraction, (from 0.01 to 5 microns), presents the real challenge. Their settling times are intolerably slow and they easily escape filtration. The behavior of colloids in water is strongly influenced by their electrokinetic charge. Each colloidal particle carries a like charge, which in nature is usually negative. This like charge causes adjacent particles to repel each other and prevents effective agglomeration and flocculation. As a result, charged colloids tend to remain discrete, dispersed, and in suspension. On the other hand, if the charge is significantly reduced or eliminated, then the colloids will gather together. First forming small groups, then larger aggregates and finally into visible floc particles which settle rapidly and filter easily.

Charged Particles repel each other



Uncharged Particles are free to collide and aggregate



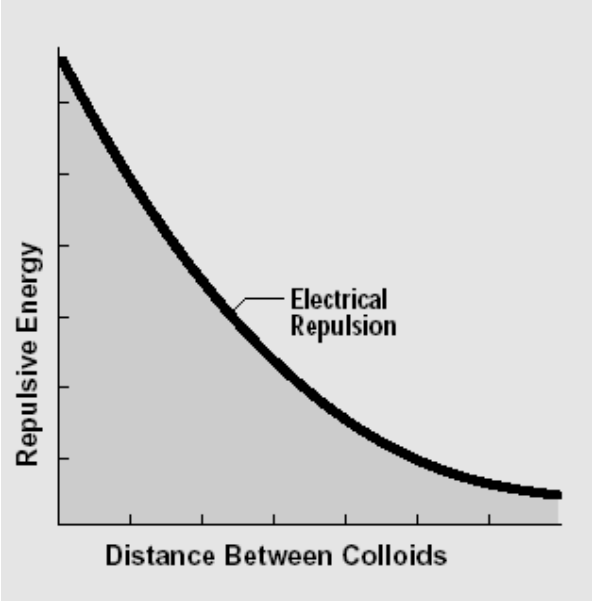
Balancing Opposing Forces

The DLVO Theory (named after Derjaguin, Landau, Verwery and Overbeek) is the classic explanation of how particles interact. It looks at the balance between two opposing forces - electrostatic repulsion and van der Waals attraction - to explain why some colloids agglomerate and flocculate while others will not.

Repulsion

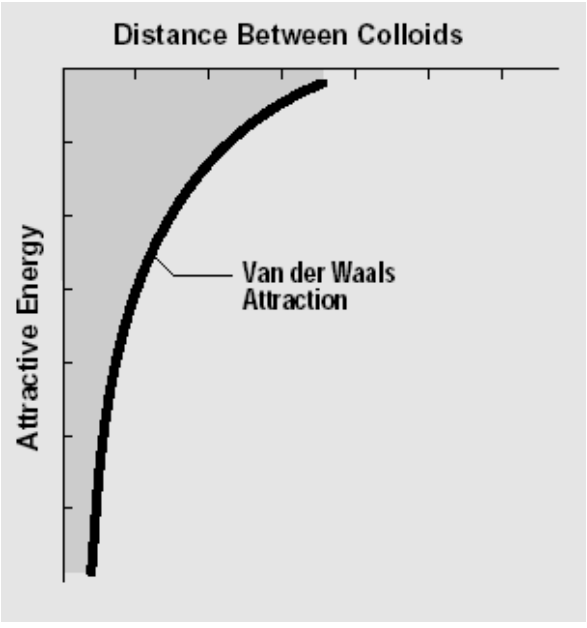
Electrostatic repulsion becomes significant when two particles approach each other and their electrical double layers begin to overlap. Energy is required to overcome this repulsion and force the particles together. The level of energy

required increases dramatically as the particles are driven closer and closer together. An electrostatic repulsion curve is used to indicate the energy that must be overcome if the particles are to be forced together. The maximum height of the curve is related to the surface potential.



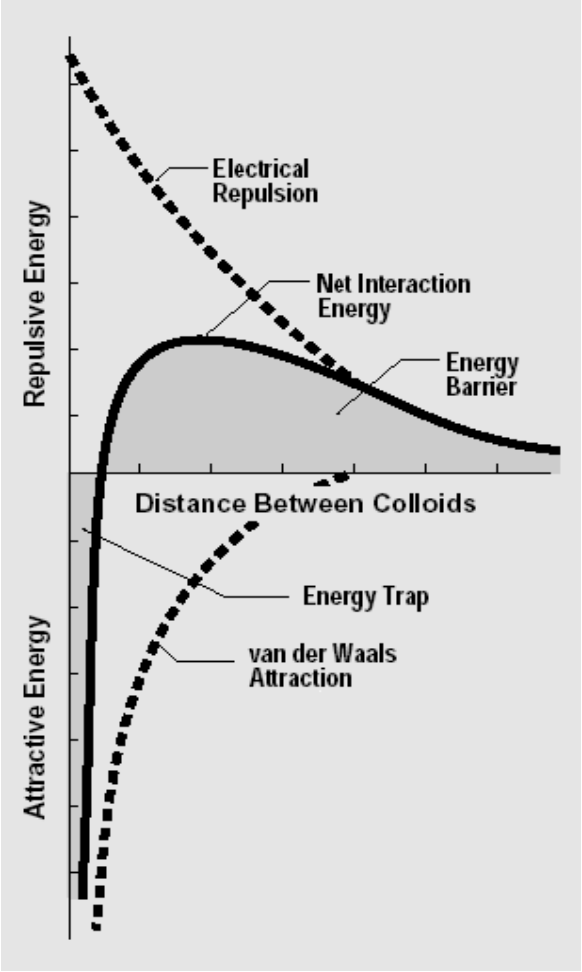
Attraction

Van der Waals attraction between two colloids is actually the result of forces between individual molecules in each colloid. The effect is additive; that is, one molecule of the first colloid has a van der Waals attraction to each molecule in them second colloid. This is repeated for each molecule in the first colloid and the total force is the sum of all of these. An attractive energy curve is used to indicate the variation in attractive force with distance between particles.



The Energy Barrier

The DLVO theory combines the van der Waals attraction curve and the electrostatic repulsion curve to explain the tendency of colloids to either remain discrete or to flocculate. The combined curve is called the **net interaction energy**. At each distance, the smaller energy is subtracted from the larger to get the net interaction energy. The net value is then plotted -above if repulsive, below if attractive – and the curve is formed.The net interaction curve can shift from attraction to repulsion and back to attraction with increasing distance between particles. If there is a repulsive section, then this region is called the **energy barrier** and its maximum height indicates how resistant the system is to effective coagulation. In order to agglomerate, two particles on a collision course must have sufficient kinetic energy (due to their speed and mass) to jump *over* this barrier. Once the energy barrier is cleared, the net interaction energy is all attractive. No further repulsive areas are encountered and as a result the particles agglomerate. This attractive region is often referred to as an energy trap since the colloids can be considered to be trapped together by the van der Waals forces.

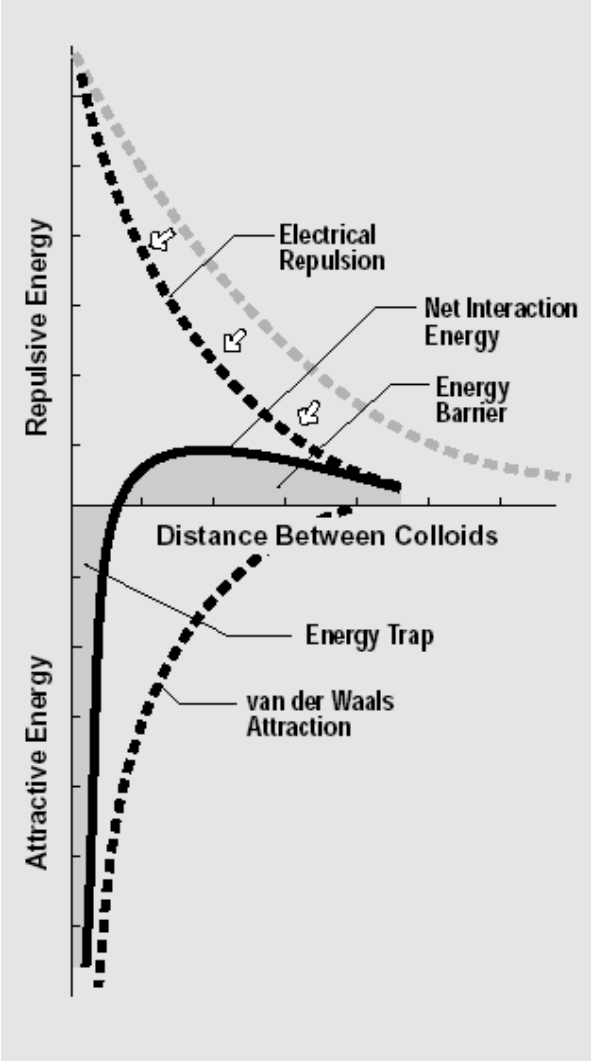


Lowering the Energy Barrier

For really effective coagulation, the energy barrier should be lowered or completely removed so that the net interaction is always attractive. This can be accomplished by either compressing the double layer or reducing the surface charge.

Compress the Double Layer

Double layer compression involves adding salts to the system. As the ionic concentration increases, the double layer and the repulsion energy curves are compressed until there is no longer an energy barrier. Particle agglomeration occurs rapidly under these conditions because the colloids can just about fall into the van der Waals “trap” without having to surmount an energy barrier.



Types of flocculants

Flocculants can be divided into three distinct categories: Inorganic, organic and natural (Kurane, et al., 1994). The most commonly used are the inorganic flocculants. The latter are a type of synthetic flocculants exemplified by aluminium sulfate, ferric sulfate and ferric chloride. Prepolymerised flocculants are used such are polyaluminium chloride (PAC) (Dampsey, et. al., 1984) and polyferric chloride (PFC) (Leprince, et. al., 1984) are more efficient. Both PAC and PFC are prepared by undertaking a partial hydrolysis of acid aluminium chloride or ferric chloride solution using a specific reactor. Another inorganic flocculant polyaluminium silicate sulfate is commercially available and it has been used in drinking water in full scale. An iron based flocculant-poly ferric sulfate or PFS has been developed. PFS is prepared by technique involving the oxidation of ferrous state iron solution under the conditions of high temperature and high pressure.

Organic flocculants have also been used extensively to enhance the flocculation of suspended solids in the treatment of process water; wastewater and effluents Important among this type are polyacrylamide and polyacrylic acid (Kurane, et. al., 1994). Organic flocculants are especially recommended for flocculating suspended solids and in non-potable raw water clarification, primary and secondary effluent clarification and oil waste clarification. Organic flocculants are also used in effluent, pulp and paper process and are important since they increase retention of fibers, fillers additives and improve drainage. Organic flocculants have high molecular weight, can work over wide range of pH, and has a low charge density and a very high molecular weight. The synthetic organic flocculants are commercially marketed in the form of dry powder, granules, beads, aqueous solutions, aqueous gels and oil in water emulsions.

Limitations of synthetic flocculants:

Despite efficiency, cost effectiveness and easy availability the synthetic flocculants their status has not been favorable currently. Most of high molecular weight flocculants are recalcitrant. Monomers of polyacralamide are potent carcinogen and neurotoxic to humans and other animals (Vanhoric *et.al;* 1983). They have detrimental effect both on flora and fauna. Natural flocculants may be composed of polysaccharide, proteins, lipids, lipoproteins, and lipopolysaccharide having a plant or microbial origin. Flocculation by naturally produced polymers involves the polymer chain sticking to multiple particles making an aggregate

large enough to settle down. There are several advantages of naturally produced flocculants; they are environmental friendly since they are biodegradable, non toxic and non carcinogenic. Besides they have high flocculating ability, low dosage requirement, diverse applications and end of pipe technologies are not required. Several non-microbial natural flocculants have been elucidated in terms of their structure and other properties; commonly used ones include starch, gums, glues, alginate etc (Singh; 2000). Some of the important natural flocculants are described below.

Starch consists of linear polymer amylose of low molecular weight (in the range of 10,000-60,000) and branched amylopectin, which is major fraction of high molecular weight (in the range of 50,000-$10^{6\ d}$ Da). The grated amylopectin is found to be a suitable flocculant for various kinds of effluents. A large number of graft co-polymers of the amylopectin have been synthesized and their flocculation efficiency evaluated in core suspension and paper mill effluents (Karmakar, et. al., 1998). Alginate has also been evaluated for flocculating abilities. A number of graft polymers of sodium alginate have been synthesized (Salehizadeh and Shojaosadati, et. al., 2002). It has better performance than many commercial flocculants (Tripathy, et. al., 1998).

Guar gum has been extensively evaluated as a flocculant in the synthetic effluent containing lead . It appeared that presence of lead ions in effluent caused the straightening effect on the polymer chains, due to this, the polymer chain fails to assume a globular form, thus making available all hydrogen bond forming sites to participate in the flocculation by bridging mechanism (Singh, et. al., 1991). Xanthum gum is yet another effective flocculant produced by *Xanthomonas compestris* and its synthesis was scaled up. Its efficiency was tested in synthetic effluent containing lead and paper mill effluent. In lead effluent, its performance is better than commercials. In paper mill effluent treatment, it acts as good flocculant and aids with alum .

Cellulose (ß-D-Glucans) is considered as food bioflocculants secreted by *Acetobacter xylimum*. It is secreted into the medium while it rapidly aggregates as a micro fibril. Bacterial cellulose acts as binder for ceramic powder and minerals and acts as thickener for adhesives. Pullulan is an (α-D-Glucans) in which small

number of maltotriose and maltotetraose units to form essentially linear. It is highly water-soluble forming viscous solution, and does not form gels and is a good adhesive. Pullulan have been used in preparation of fibers and acts as good thickening agent (Nell, et. al., 1990). The regular alternation of α-1, 4 and α-1, 6 bonds results in two distinctive properties, structural flexibility and enhanced solubility (Leathers, et. al,. 1993). These properties suggest that Pullulan may be used for both medical and industrial purposes (Le Dury, et. al., 1998). Pullulan produces high viscosity solutions at relatively low concentrations and can be utilized to form oxygen impermeable films, thickening or extending agents, or adhesives (McNell and Kristiansen; 1990). Gellan polymers carries o-acetyl and glycerol subsistent on a linear polymer of 500 KDa that is composed of tetrasaccride repeat units. The acyl groups inhibit crystallization of localized region of gellan chain and weak elastic thermo reversible gels are formed.

Bioflocculant production from lower molecular fatty acids

Biolocculant production from lower molecular fatty acid can be an innovative strategy for utilizing waste sludge digestion liquor (Fujita *et.al;* 2001). Studies on the production characterization and application of a novel bioflocculant was reported in a *Citrobacter* sp. Tk F04, which was screened out of 1,564 natural isolates. This strain is capable of bioflocculant production from acetic acid and propoinic acid; the bioflocculant could be easily recovered from the culture supernatant by the ethanol precipitation. Fed batch cultivation with this bioflocculant was found to be effective for a flocculating a kaolin suspension when added at final concentration of 1-10 mg/L over a wide range of pH (2-8) and temperature of 3-95^0 C. This novel bioflocculant was able to flocculate a variety of inorganic and organic suspended particles including kaolin, diatomite, bentonite activated carbon and sludge with superior efficiency tan inorganic flocculants.

Bioflocculant producing algae and fungi

Blue green algae have been reported to produce bioflocculants during the stationary phase in batch cultures. Prominient species were reported to be *Chalmydomonas maxicana* and *Anabaena* sp. ATCC33047, which produced bioflocculant of polysaccharide in nature. Chemical analysis of polysaccharide depicts the characteristics of heteropolysaccharide. It consists of Xylose, glucose,

galactose and mannose. The infrared spectrum of exopolysaccharide showed absorption bands of carboxylate group. The average molecular mass of this polymer was 1.35 MDa. Aqueous dispersion at exopolysaccharide concentration ranging from 0.2% to0.6% (w/v) showed marked sheer thinning properties. Fewer studies however, on fungal flocculant has been done, among the fungal strains isolated, a strain of *Aspergillums sojae* (Nakamura, et. al., 1976) was shown to produce significant amounts of biofocculants.

Bioflocculant producing Bacteria

Several bacterial strains have been reported to produce flocculants. Salehizadeh, et. al*.,* (1999) reported a *Bacillus sp*. As –101 capable of producing flocculating substances. The latter was partially purified by cold ethanol and cetyl pyridinium chloride. It acted under acidic conditions and it's maximum flocculating activity was observed at pH 3.5.It's flocculating activity was stimulated by the addition of Al^{3+}, Fe^{2+}, Ca^{2+} in the optimum concentrations of 0.2 to 25.8mM respectively.

Suh, et. al., (1997) reported a *Bacillus* sp. (DP –152) isolated from soil samples, thiscstrain produced excellent flocculating substances. It was purified by ethanol precipitation and cetyl pyridinium chloride precipitation and gel permeation chromatography. The bioflocculant DP-152 was estimated to have a molecular weight of over $2x10^6$ Daltons.

Bacillus licheniformis CCRC 12826 was found to be producing an extracellular biopolymer flocculant in the large quantity (Shih, et. al., 2001). The bioflocculants had a molecular weight over $2x10^6$ KDa, and could be easily purified from culture medium by ethanol precipitation. The bioflocculant efficiently flocculated various organic and inorganic suspensions. Its flocculating activity was synergistically stimulated by the addition of cations Ca^{2+}, Fe^{2+}, Al^{3+}. and was most effective at neutral pH ranges. The bioflocculant was characterized to be polyglutamic acid. *Bacillus firmus* a soil isolate produced a strongly acidic, polysaccharide-flocculating agent (Salehizadeh *et.al;* 2001). Bioflocculant produced by this species was thermo stable and it's 48% of the activity remained after heating at 100^0C for 50 minutes. It acted on both inorganic and organic suspensions such as kaolin, activated carbon, pigments and yeast. Its flocculating activity with kaolin suspension was stimulated by Ca^{2+}, Mg^{2+} and Fe^{2+}. The bioflocculant was purified to homogeneity by ethanol and cetyl pyridinium chloride precipitation and

consisted of glucose, fructose, mannose, galactose in the molar ratio of 12:1, 5:7, 3:1,1; 1 with the molecular weight of approximately $2x10^6$ Da.

Rhodococcus erytropolis S-1 produced a flocculant for various colloidal suspensions and colored pigments (Kurane, et. al*.,* 1986 and Takeda, et. al., 1991), *Nocardia amarae* YK1 Takeda, et. al., 1992) and *Paecilomyces* sp (Takegi and Kadowaki.,1985) produced a protein flocculant. The flocculants produced by *Arcuadendron sp*. Ts-4 (Lee, et.al.,) and *Aratrobacte*r sp.(Wang,et.,al.,1995) were glycoprotein. *Bacillus mucilaginosus* was found to produce bioflocculant (Deng, et. al*.,* 2003). It had a good flocculating capability and could achieve a flocculating rate of 99.65% for kaolin suspension at a dosage of only 0.1 ml/L .The major components of flocculant were mainly of uronic acid (19.1%), neutral sugar (47.4%) and amino sugars (2.7%) .

The exopolymer secretion by *Zoogloea* MP6 and flocculation by *Zoogloea* MP6 has been observed .Polymer formation was initiated in mid logarithmic growth phase and quantity produced appeared to be influenced by the level of carbon and nitrogen in the culture medium. Exopolymer obtained from *Zoogloea* strains and activated sludge flocs have been chemically characterized and amino sugars were found to the major components (Tyzuka, et. al., 1973,.Farrah, et. al*.,* 1974).In this the amino sugars was used as an indirect measure of exopolymer formed by *Zoogloea sp*. MP6 in experiments conducted to demonstrate the relationship between the polymer production and flocculation.
The synthesis of exopolymer by *Aureobasidium pullulans* ATCC 42033 was reported by Lee, et. al., (1999). It was cultured under aerobic conditions with glucose, mannose and glucose analogs as energy source. The exopolymer extract produced under these conditions were composed of glucose and mannose. The glucose content of exopolymer extract formed with glucose and mannose as the carbon source was between 91% and 87% as the culture time increased the glucose content of exopolymer extract formed with glucosamine decreased to 55, 29 mol % and molecular weight increased from $2.73\ x10^6$ to $4.86\ x10^6$ KDa.

The production of exopolymer by *Zoogloea ramigera* caused flocculation of activated sludge (Butterfield, et. al., 1935 and Heukelekian, et. al., 1939).

Exocellular polymer was isolated from *Zoogloea strain* 106 and activated sludge flocs by blending samples with phosphate buffer and precipitation of solubilized polymer with cetyl trimethyl ammonium bromide. Samples of polymer from these sources were similar and yielded amino sugars as the principal components after acid hydrolysis.

Flocculation of fine fluorite particles was studied in bacterium *Corynebacterium xerosis* (Hass *et. al*; 1999). It was found that *Corynebacterium xerosis* cell adhere to the fluorite surfaces promoting the aggregation of the particles. High quality flocs can be obtained rapidly at pH 7 using a cell concentration of 40 mg/l.

Biosorption of heavy metals by thermo tolerant polymer producing bacterial cells and the bioflocculant were demonstrated by Songklanakarin, et. al*., (*2002). Three strains of thermo tolerant polymer producing bacteria - *Bacillus subtilis* WD90, *Bacillus subtilis* SM29 and *Enterobacter agglomerans* SM38 as well as their bioflocculants were used to investigate adsorption of heavy metals (nickel and cadmium). The effect of pH was investigated on adsorption of heavy metals by the biopolymeric flocculants.

Another isolate of *Enterobacter* sp produced a bioflocculant under batch and fed batch cultivation. The effect of medium composition on the production of polymer from the isolate was investigated. Among the carbon sources (1%) tested, galactose and sucrose gave higher polymer yield (2.50 and 2.45 g/l respectively).Compared to maltose, fructose and glucose. Sucrose was chosen due to its lower cost, optimum concentration was found to be 3% giving the polymer yield of 2.72 g/L after 72 hour cultivation. Several sources of inorganic nitrogen and organic nitrogen tested had no influence on polymer yield. The optimum concentration of yeast extract was 0.005%.The optimum initial pH was 7 and higher yield was obtained under controlled pH 7.Further increase of polymer concentration could be achieved from fed batch culture with the addition of 10% sucrose every three days of cultivation. The polymer yield increased to 6.1 g/L, which was 2.5 times higher than initial volume.

A bioflocculant produced by marine *Myxobacterium nannocystis* sp. NU-2. This strain is isolated from salt soil sample collected from the Coast of Huanghai Sea,

china. Morphological properties and 16s rDNA sequence analysis indicated that isolate is a novel species related to genus *Nannocystis* NU-2 also produced a new kind of flocculating substance in the starch medium with a yield of 14.8 g/L. It was composed of 40.3% proteins and 56.5 % polysaccharide of which glucose, mannose and glucouronic acid were the principal constituents in the relative per portions of 5:4:1 .The flocculation activity of NU-2 depended strongly on cations such as Fe^{2+} and Al^{3+}. It had high flocculating activity values of 90% which remains unchanged over an extensive pH range (2-13). Flocculant was tested for its ability to bleach dyeing liquors and its bleaching activities were 98.25 % for acid red in 100mg/l of the flocculant and 99.5% for direct emerald blue in 50mg/l of the flocculant under the test condition.

MATERIAL AND METHODS

3.1 Collection of wastewater samples from different industries

The wastewater samples were collected from different industries (please see Table 1 for source details) in screw cap sterilized bottles and analyzed within 2 –3 hours from collection in the laboratory. For enrichment approximately 10 % of the samples were inoculated in Flocculant isolation broth medium (FIB and incubated at 28^0C, with shaking (120 rpm) for 24 hours. Following incubation, serial dilutions were made up to 10^{-8} in sterile saline water (0.85%) and 100µl of appropriate dilutions were plated on to nutrient agar plates. All plates were incubated at 37^0C for 24-48 hours. Colonies obtained were counted on colony counter.

3.2 Isolation and Purification of the biopolymer bacteria

For isolation and purification of bacteria, initial screening was carried out by kaolin assay (Suh, et. al., 1997) Out of many bacteria, which flocculate kaolin suspension were selected for further studies. The process of Kaolin assay for the flocculating activity is mentioned below:

Kaolin assay for checking the Fractional Flocculation and flocculating activity

Kaolin clay (Hi Media, Mumbai) was used as suspension materials for estimating the flocculating activity. In this 10 ml $CaCl_2$ (10%), 0.5ml culture and 9.5ml of distilled water were added to 80ml of kaolin solution (5g/L). The pH was adjusted to 7 and solution mixed gently that solution was allowed to stand at room temperature for 5 min. Decrease in turbidity of the upper phase at 550 nm with a spectrophotometer was reported. The Fractional Flocculation (%) and the Flocculation activity was calculated by the following equation respectively (Kurane, et. al., 1994).

$$\textbf{Fractional Flocculation (\%) = (B-A) *100 / B}$$

$$\textbf{Flocculation Activity=1/A –1/B}$$

Where A – Optical density of sample at 550 nm

B – Optical density of reference at 550 nm.

The two isolates RDL1 and RDL2 possessing maximum flocculating activity were used for further studies.

Name of the Industry	**Name of isolates**	**Source**
Hindustan Lever Ltd. Rajpura.	HL1	Activated Sludge
	HL2	Activated Sludge
Milk Plant Ltd. Bahadurgarh.	SG1	Activated Sludge
	SG2	Activated Sludge
	SG3	Activated Sludge
Chemica Factory Patiala.	ST1	Activated Sludge
	ST2	Activated Sludge
Rainbow Denim Ltd. Lalru.	RDL1	Activated Sludge
	RDL2	Activated Sludge
	RD3	Activated Sludge
	RD4	Activated Sludge
	RD4B	Activated Sludge
Amrit Banaspati Company Ltd.Rajpura	SS1	Activated Sludge
	SS2	Activated Sludge

Table 1: Bacterial strains isolated from different industries

3.3 Growth Kinetics of two isolates Isolate 1 and Isolate 2

Isolate RDL1 and RDL2 2 were grown at 28^0C, with shaking (120 rpm) in FIB media for 24 hours. Aliquots (5 ml) were withdrawn after every 6 h ours and the absorbance measured at 550nm. Flocculating activity (kaolin assay) of Isolates RDL1 and RDL2 was checked after every 6 hours and was continued for 60 hours.

3.4 Optimization of cultural parameters

Effect of growth and flocculating activity was studied on the two bacteria for the following:

(a) Carbon sources

Isolates RDL1 and RDL2 were inoculated in FIB media containing different carbon sources such as Glucose, Sucrose, Fructose, and Starch and incubated with shaking (120 rpm) at 28^0 C. Growth profile of both was studied within the intervals of 2 hours up to 24 hour. The flocculating activity was studied after every 6 hours and was continued for 60 hours.

(b) Inorganic Nitrogen sources

Isolates RDL1 and RDL2 were inoculated in FIB media containing different inorganic nitrogen sources such as ammonium sulfate and ammonium chloride and ammonium phosphate in 250 ml flasks and these were incubated at 28^0C with shaking at (120 rpm). Growth kinetics was studied within the intervals of 2 hours up to 24 hour and flocculating activity was studied after every 6 hours and was continued for 60 hours.

(c) Organic Nitrogen sources

Isolates RDL1 and RDL2 were inoculated in FIB media media containing different organic nitrogen sources Urea, Beef extract, and Peptone in 250 ml flasks and these were incubated at 28^0C with shaking (120 rpm). Growth kinetics was studied within the intervals of 2 hours up to 24 hour and flocculating activity was studied after every 6 hours and was continued for 60 hours.

(d) Cations

Kaolin assay was done with the various cations to check the maximum flocculating activity of Isolates RDL1 and RDL2

(e) pH

pH of FIB media was adjusted to 6, 7.5, 10.5 by using 10 N HCl and 10 N NaOH. Media was inoculated with Isolates RDL1 and RDL2, incubated at 28^0C with shaking (120 rpm). The kaolin assay was done to check the maximum flocculating activity, after 24 hours.

(f) Static conditions

The inoculated culture of Isolates RDL1 and RDL2 were kept in static conditions (without aeration). At different time intervals, the flocculating activity was checked.

(g) Stress conditions

Isolates RDL1 and RDL2 were inoculated in FIB media lacking carbon source, Inorganic nitrogen source and organic nitrogen source in FIB media and then flocculating activity was checked after incubation at 28^0C with shaking (120 rpm).

(h) Effect of supernatant and pellet

The grown culture of Isolates RDL1 and RDL2 2 were centrifuged. Kaolin assay was done to with .5 ml of supernatant and flocculating activity was checked. Pellet was resuspended with 1 ml of saline solution (0.85%) and the flocculating activity was checked with this 0.5ml of resuspended culture**.**

3.5 Isolation and partial purification of bioflocculants

In order to purify the bioflocculant the isolates were grown in the 250 ml flasks for 24 h at 28^0C with shaking at 15000 rpm for 20 minutes to remove the cells debris. The supernatant was concentrated; ethanol was added (approximately double) to the concentrated supernatant and was dried in a desiccators overnight. Thereafter the crude bioflocculant was dissolved in distilled water, 2% (w/v) cetylpyridinium chloride (CPC) added until no more insoluble CPC bioflocculant complex was formed. The culture was centrifuged and the precipitate dissolved in saline solution (0.85%) washed with cold ethanol thrice and bioflocculant powder weighed and preserved in airtight vials.

3.6 Analysis of purified bioflocculants

3.6.1 Protein content

In order to test whether the flocculants contain protein molecule, the portion of the lyophilized bioflocculant was analyzed using Folin-Lowry method with bovine serum albumin as standard.

Bovine Serum Albumin (BSA) was used as standard in different concentrations (100 µg/ml, 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, and 1000 µg/ml and purified product in the range of 5 mg/ml and 1 mg/ml in duplicates. 50 ml solution of (2g of Na_2CO3 + 0.1M NaOH) and 50 ml solution of (0.5g per 100ml of $CuSO_4$ + 1g per 100ml of Na-K-tartarate) was added. 5ml Folin reagent was added to it and incubated for 30 minutes in dark. Absorbance was recorded at 750 nm. The amount of total protein present in sample was calculated from calibration curve prepared using pure BSA as standard.

3.4.2 Sugar content

The total carbohydrate content of the flocculant was determined by phenol sulfuric acid method as described by Chaplin and Kennedy (1986).below:

200mg of sample was added in hot 80% Ethanol to remove sugar. This was centrifuged (12000 rpm, 3 min) and residues retained. Residues were washed repeatedly with hot 80% ethanol till the washing did not give color with anthrone reagent. The residues were dried well over a water bath. To the residues 5ml of water and 6.5ml of 52% perchloric acid was added. Extract was kept for 20minutes at 0^0C. The latter was centrifuged (12000 rpm, 3 min) and supernatant

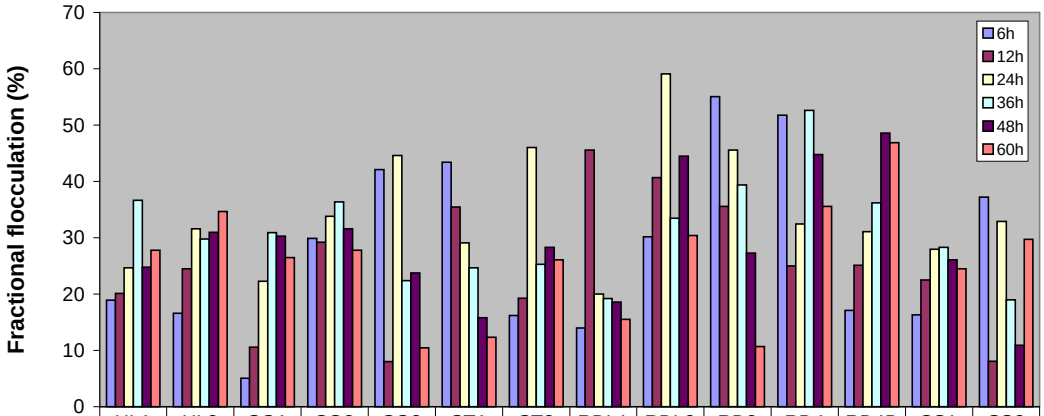
was saved. The extraction was repeated by using fresh perchloric acid. The supernatant was centrifuged and pooled and made up to 100ml. 0.2, 0.4, 0.6, 0.8, 1ml was pipetted out from glucose standard (100mg in 100ml of water) into the series of test tubes. 0.1, 0.2ml of the sample solution was pipetted out in two separate test tubes. The volume was made up to 1ml with water, in each tube. A blank was set with 1ml of water. 1ml of phenol solution was added to each tube. 5ml of 96% of Sulfuric acid was added to each tube and shaken well. After 10 min the contents in tubes were shaken and placed in water bath at 25-30^{0}C for 20 min. Color was read at 490 nm. The amount of total carbohydrates present in the sample solution was calculated from standard graph (prepared using glucose).

RESULTS & DISCUSSIONS

1. Screening of flocculant- producing bacteria

Since flocculating bacteria may have predominance in wastewater or sludge samples, a number of these sources were used for the purpose of isolation. For the screening of novel flocculant producing bacteria 14 bacterial strains were isolated that excreted mucous material on the agar plate of the screening medium were isolated from various industrial wastewater and sludge the bacteria isolated after enrichment from various sources. The predominant isolates were selected from plates of dilution and restreaked onto nutrient agar plates. Each isolate were further screened preliminarily on basis of their flocculating ability prior to growth both in nutrient broth and Flocculant isolation broth. The culture broth of each isolate was tested for its ability to flocculate Kaolin clay. Maximum flocculating activity was detected in two of the isolates obtained from textile dying industry named as Rainbow Denim Ltd. Located at Lalru in the state of Punjab.

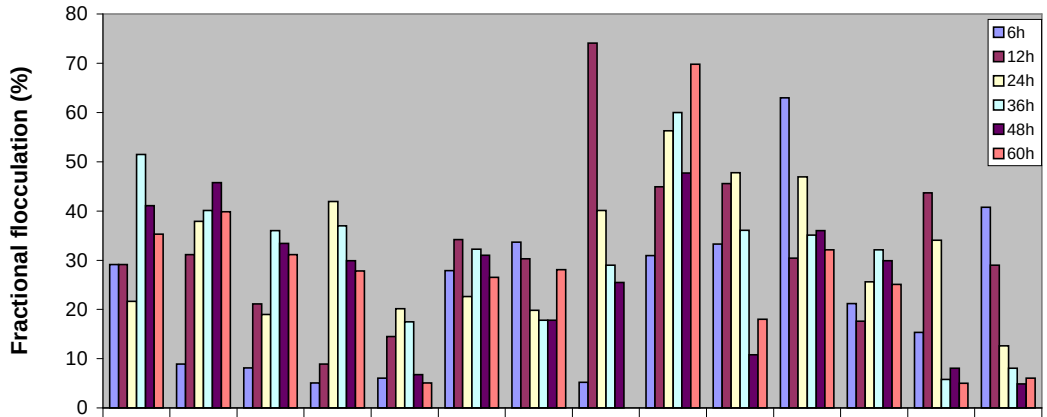
Fig. 1a & fig 1b shows time course study of the flocculant activity in the 14 isolates after enriching them both in minimal mineral media called as flocculant isolation broth and rich media that is nutrient broth. However the maximum flocculant activity was observed in the two Strains namely RDL1 and RDL2 after 12^{th} and 60^{th} hours respectively, grown in minimal mineral media only. Same Culture grown in Nutrient Broth did not Contribute to Significant Flocculation activity,



	HL1	HL2	SG1	SG2	SG3	ST1	ST2	RDL1	RDL2	RD3	RD4	RD4B	SS1	SS2
6h	18.9	16.61	5.05	29.9	42.11	43.4	16.2	14	30.2	55.1	51.8	17.1	16.3	37.2
12h	20.1	24.5	10.6	29.2	8.03	35.5	19.3	45.6	40.7	35.6	25	25.1	22.5	8.05
24h	24.7	31.6	22.3	33.8	44.6	29.1	46	20	59.1	45.6	32.49	31.1	28	32.9
36h	36.7	29.8	30.9	36.36	22.4	24.7	25.3	19.2	33.5	39.4	52.59	36.2	28.3	19
48h	24.8	31	30.3	31.6	23.75	15.82	28.3	18.6	44.5	27.3	44.8	48.6	26.1	10.9
60h	27.8	34.7	26.5	27.8	10.5	12.36	26.1	15.5	30.4	10.7	35.6	46.9	24.5	29.7

Bacterial Isolates

Fig 1a.



	HL1	HL2	SG1	SG2	SG3	ST1	ST2	RDL1	RDL2	RD3	RD4	RD4B	SS1	SS2
6h	29.1	8.92	8.1	5.05	6.06	27.9	33.7	5.16	30.9	33.3	63	21.2	15.3	40.8
12h	29.1	31.1	21.1	8.9	14.48	34.15	30.3	74.1	44.9	45.6	30.4	17.6	43.7	29
24h	21.6	37.9	19	41.9	20.13	22.6	19.8	40.1	56.3	47.8	46.9	25.6	34.1	12.6
36h	51.5	40.1	36	37	17.5	32.23	17.8	29	60	36.1	35.11	32.1	5.76	8.06
48h	41.1	45.77	33.4	29.93	6.77	31	17.8	25.5	47.7	10.8	36	29.9	8.08	4.87
60h	35.3	39.8	31.1	27.8	5.05	26.5	28.1		69.8	18	32.1	25.1	5.01	6.06

Bacterial Isolates

Fig.1b

2. Distribution of flocculating component

Fig.2a. & Fig.2b shows the distribution pattern of flocculating components in culture broth of Isolates RDL1 and RDL2. was examined .Both the strains were cultured in flocculant isolation broth for 36 hours and 72hours respectively. Most of the flocculating activity was found in the cell free supernatant. The results are in consistent with the earlier observations of the studies carried out with other stains elsewhere (Suh, et. al., 1997) Fig. 2c show the growth profile of the Isolates RDL1 and RDL2. The maximum flocculant producing activity was observed in 12 hours during the log phase in case of isolate RDL1. Where as in case of isolate RDL2 flocculant production was not parallel to the cell growth and a large amount of flocculant was released into the culture in death phase or when the bacteria has ceased to grow.

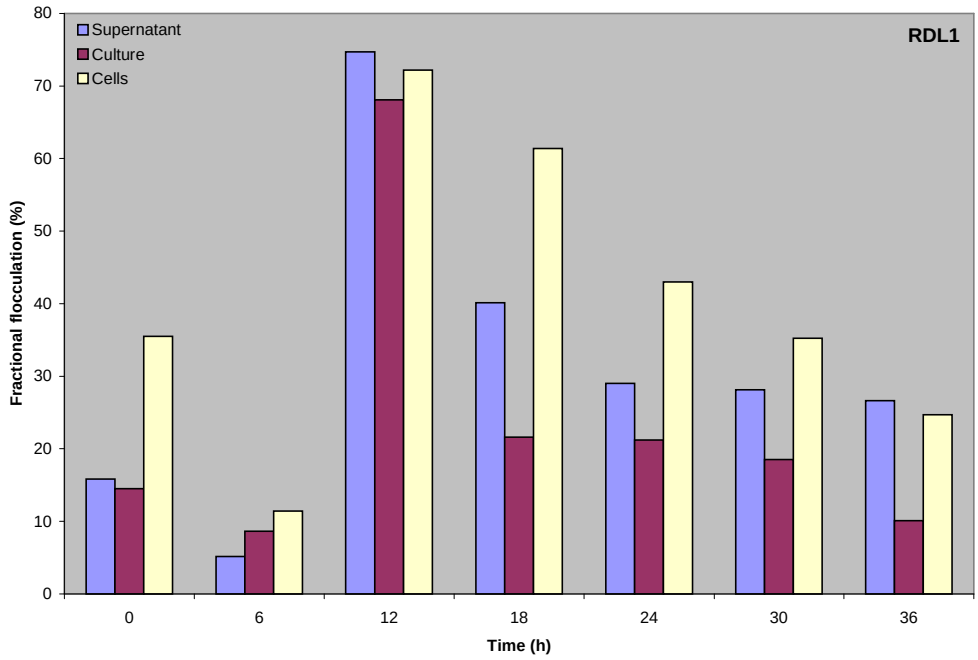


Fig. 2a

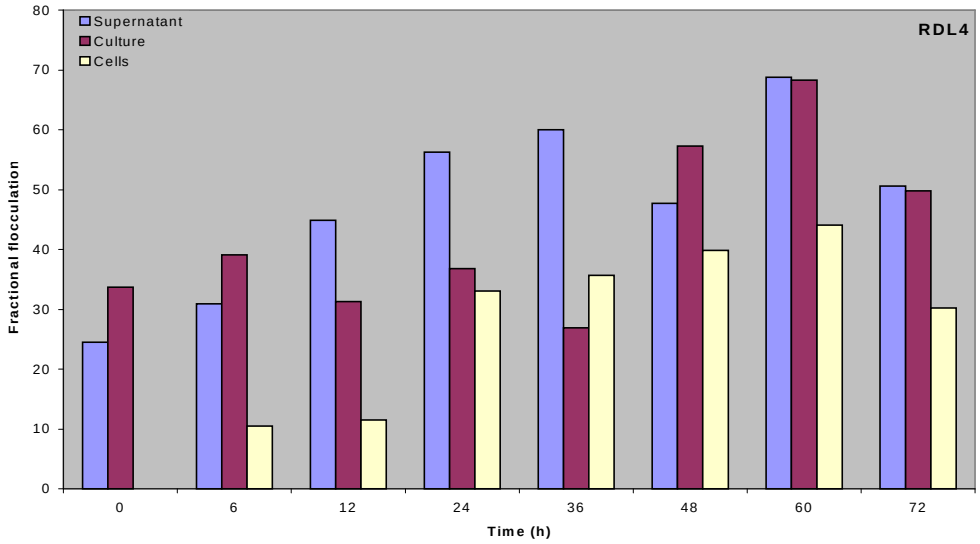

Supernatant
Culture
Cells
RDL4
Fractional flocculation
Time (h)
0
6
12
24
36
48
60
72
10
20
30
40
50
60
70
80

Fig: 2b.

.

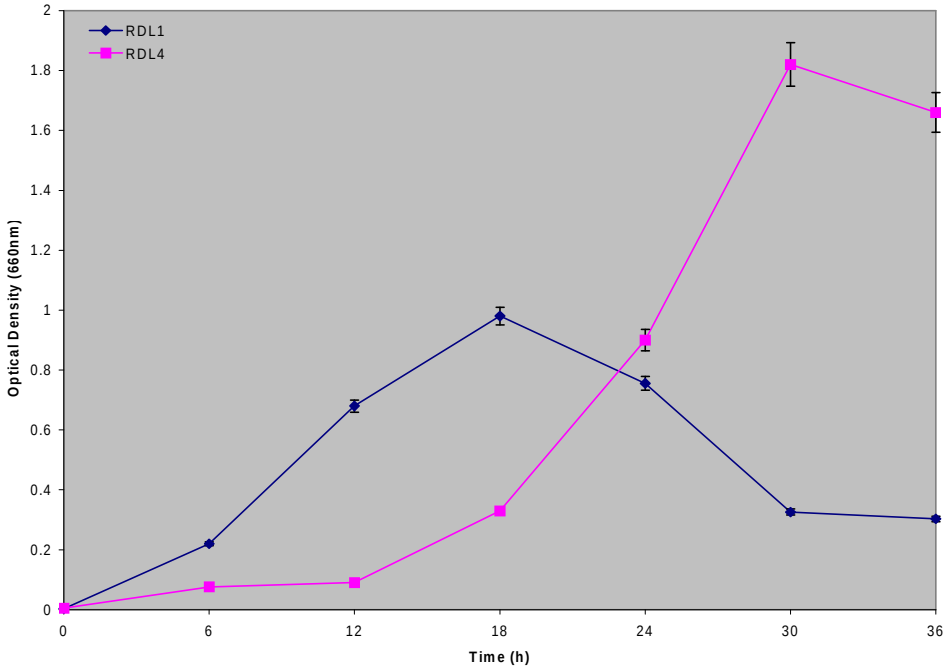

RDL1
RDL4
Optical Density (660nm)
Time (h)
0
6
12
18
24
30
36
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2

Fig. 2c

3. Effect of different cultural parameters and other factors on bioflocculant production

3.1 Effect of different carbon sources

Among the various carbon sources tested in case of both Isolates RDL1 and RDL2 glucose and fructose were effective for the flocculant production. These two sugars component appeared favorable for cell growth as well. While on the other hand sucrose and starch can show flocculant production, But less as compare to the glucose and fructose. but Sucrose and starch are not favorable for growth. The maximum flocculant production were recorded at the during the log phase in case of strain RDL1 While In case of strain RDL2, also glucose and fructose are very much effective for the flocculant production, starch and sucrose can show flocculant production but their rate is low as compare to the glucose and fructose

as shown in Fig.3a.

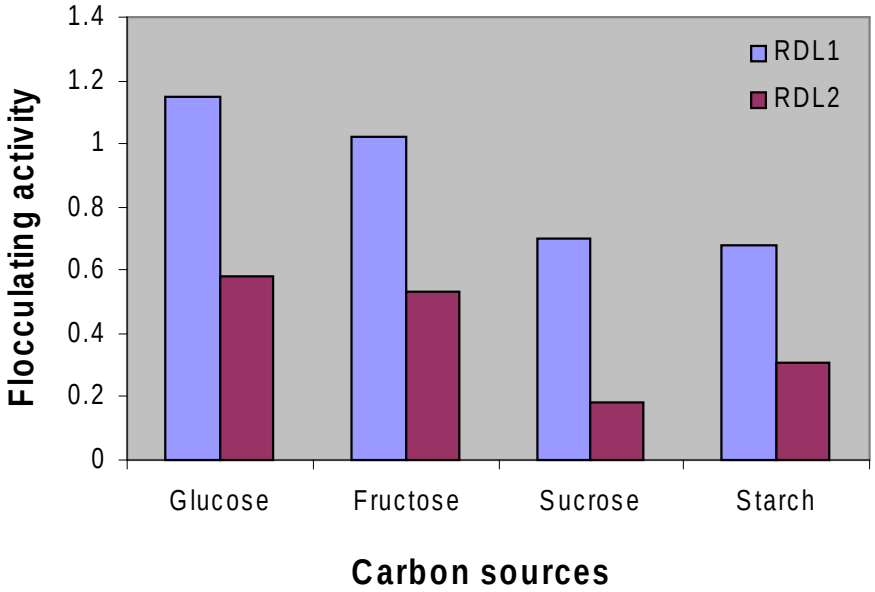


Fig. 3a

3.2 Effect of different nitrogen sources

Among the inorganic Nitrogen source tested in case of strain RDL1, Ammonium sulfate and ammonium phosphate appeared favorable for both flocculant production and cell growth. But on the other hand ammonium chloride retarded

cell growth and has very less flocculating activity as compared to the ammonium sulfate and ammonium phosphate. While In case of isolate RDL2, among the inorganic nitrogen source tested ammonium sulfate appeared most favorable for maximum flocculant production. While the ammonium phosphate and ammonium chloride can produced very less flocculant (Fig.3b). There were three Organic Nitrogen sources among them urea and peptone appeared favorable for both cell growth and flocculant production. Where as in beef extract cell growth was less, and less flocculation activity was observed (Fig.3c).

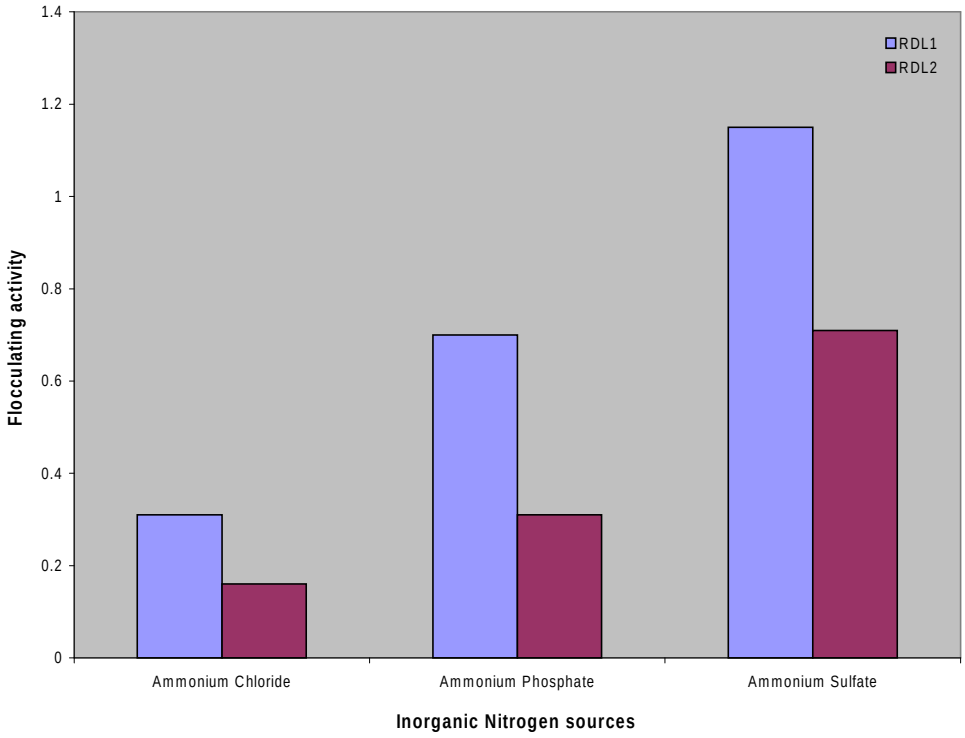


Fig.3b

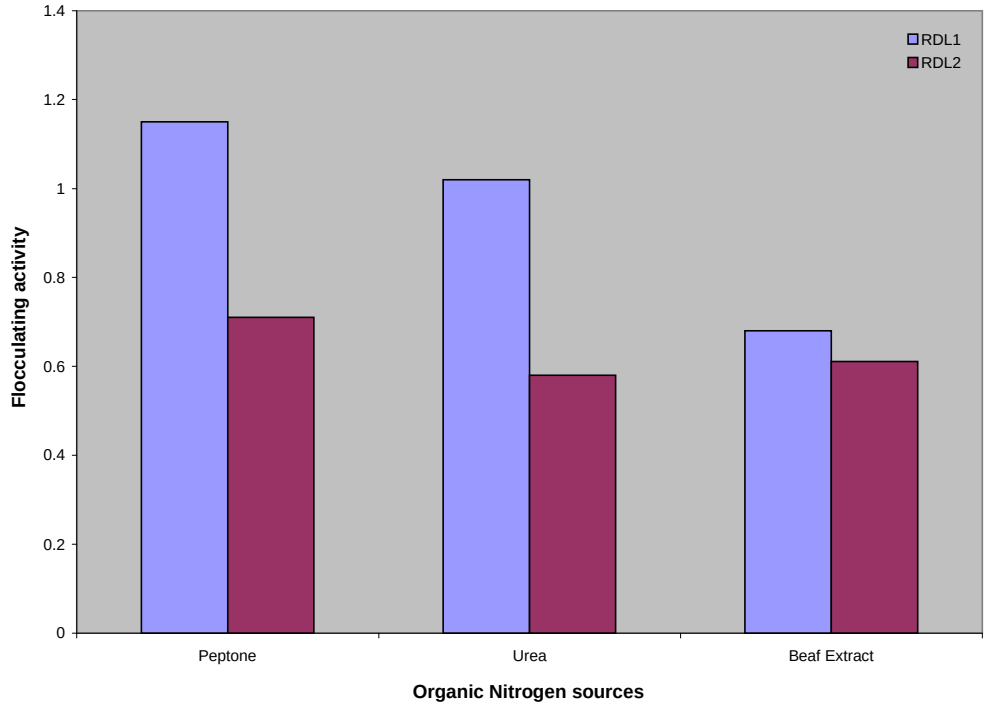


Fig 3c

3.3 Effect of cations to flocculating activity

In case of both the isolates i.e., RDL1 and RDL2, amongst the various cations tested Ca^{2+} show maximum flocculating activity, while the other ions like Mg^{2+} , show less flocculating activity. Whereas in case of Na^{+} Proved to be very poor in showing flocculating activity. The results are shown in (fig.3d). The results indicates the role of cations in the bioflocculation process. The divalent cations ca^{2+} and Mg^{2+} have nearly equal capacity to biofloculate, where as Na^{+} have detrimental effect on bioflocculation Process. therefore the above study revels that Cations are an important factor in determining Floc properties.

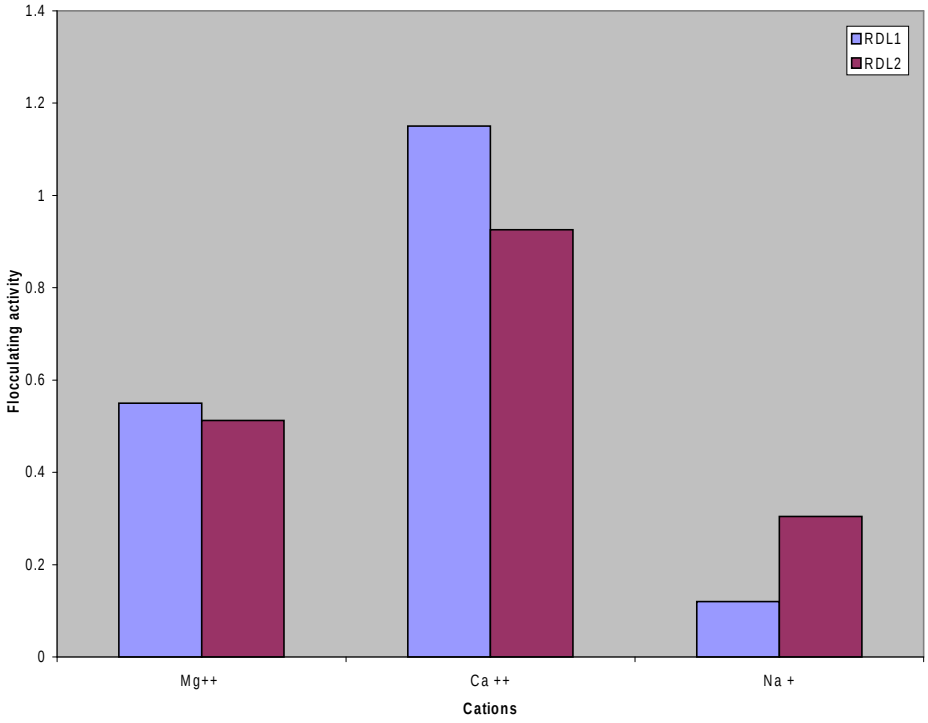


Fig.3d

3.4 Effect of initial pH

The Flocculant production were affected by the initial pH. In case of RDL1,the alkaline pH 7.5 greatly stimulated the flocculant production. While at the acidic pH 6 and highly alkaline pH 10.5, the flocculant production was very less as compare to that obtained at pH 7.5. As shown in fig.3e.Similar results were found in the isolate RDL2. There was no significant variation on the effect of pH in the Strain RDL2 when compared to strain RDL1.

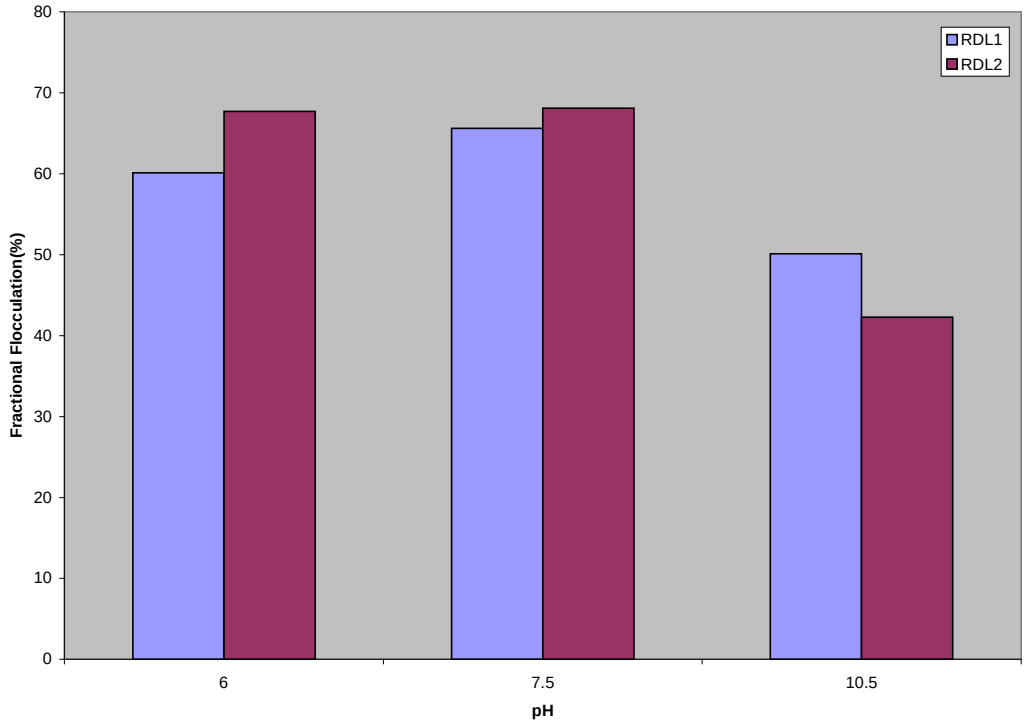


Fig. 3e

3.5. Effect of Stress Conditions

(Fig.3f).shows a comparison between the flocculant producing profile of the two isolates RDL1 and RDL2 under different stress conditions of carbon, and nitrogen sources. The flocculant production In case of isolate RDL1, the rate of production of the flocculant greatly gets reduced when glucose as carbon source was not provided in the culture medium Also when inorganic and organic nitrogen sources viz. Ammonium sulfate and peptone were not provided in the culture medium simultaneously the rate of flocculant production decreases. In case of isolate RDL2 also, the flocculant production was decreased under stress of carbon, Inorganic nitrogen and Organic nitrogen sources. This effect of stress condition were checked as it was mentioned by (Bura et al,1998),microorganisms produces higher amount of flocculants when they are under nutritional stress.

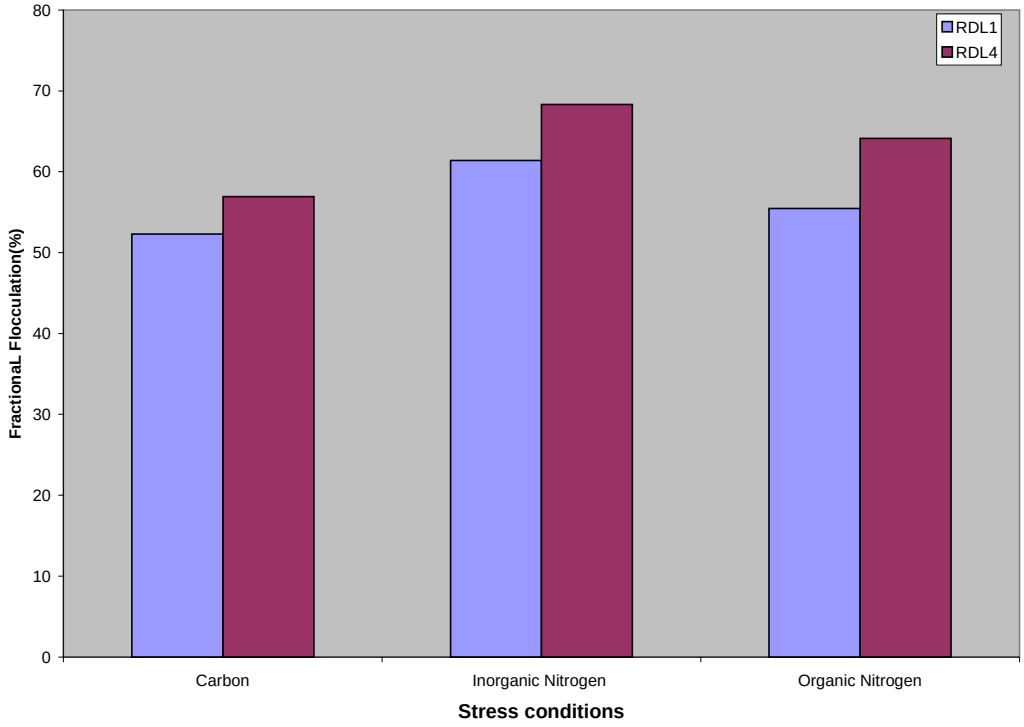


Fig. 3f.

3.6. Effect of Static condition

When flocculating activity of both the cultures were compared under shaking and non shaking conditions it was found that both the strains RDL1 and RDL2 exhibited much less flocculating activity under non shaking conditions as shown in (fig.3g). Sometimes due to agitation of the culture medium (salehizadeh 2001 et al) Yield of polymeric flocculant production by bacterial cells might be greatly reduced there fore study was conducted to check the effect of bioflocculant production and measured in terms of flocculant activity under shaking and non shaking condition.

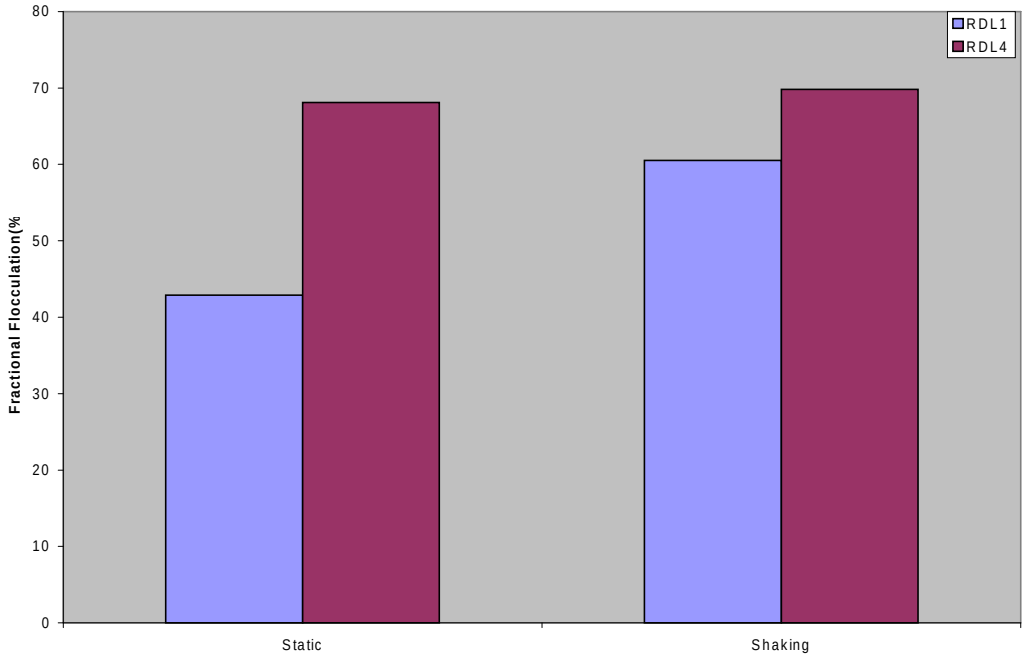


Fig. 3g. Compression between static and shaking condition of the strain RDL1 and RDL2.

4. Physicochemical properties of partially purified bioflocculant

The yield of the bioflocculant obtained after partial purification by cetyl peridium chloride(CPC) treatment and simultaneous ethanol precipitation was higher in case of isolate RDL1 as shown in Table. II

As shown in Table II both protein and carbohydrate residues were present in the bioflocculant. The total carbohydrate was more than that of protein content in both the isolates RDL1 and RDL2. It suggests a predominance of carbohydrates in the bioflocculant fractions. When the carbohydrate and protein content was compared the carbohydrate content was significantly higher in case of isolate RDL1 whereas the protein content was higher in case of RDL2

Table II: Physicochemical properties of partially purified bioflocculant

Sl. No.	Dry wt. purified bioflocculants	Total protein content	Total carbohydrate content
RDL1	1.08g/l	1.29mg/l	92.1mg/l
RDL2	0.88g/l	3.59mg/l	12.5mg/l

As shown in Table III the flocculating activity of partially purified bioflocculant was approximately two folds higher when compared with crude extracts in the culture medium. The bioflocculant purified from strain RDL 1 exhibited flocculating activity of 91% when one gram equivalent of the biopolymeric flocculant was used. The flocculant obtained after partial purification from the isolate RDL4 was lesser then but yet significantly high.

Table III. Flocculating activity of bioflocculant

Bacterial Isolate	Yields of partially purified bioflocculant	Flocculating activity (%)	
		5mg/ml	1g/ml
RDL1	1.08g/1000ml culture.	88%	91%
RDL4	0.88g/1000ml culture.	77%	82%

ANNEXURE - I

Annexure 1

1. FLOCCULATING BROTH MEDIUM:

Composition

Ingredients	g/l
Peptone	5.0
Ammonium sulfate	2.0
Yeast extract	1.0
Calcium chloride di hydrate	0 .7
Sodium chloride	0 .1
Magnesium sulfate	0.2
Dipotossium hydrogen phosphate	1.0
Glucose	1.0
Agar	3.0

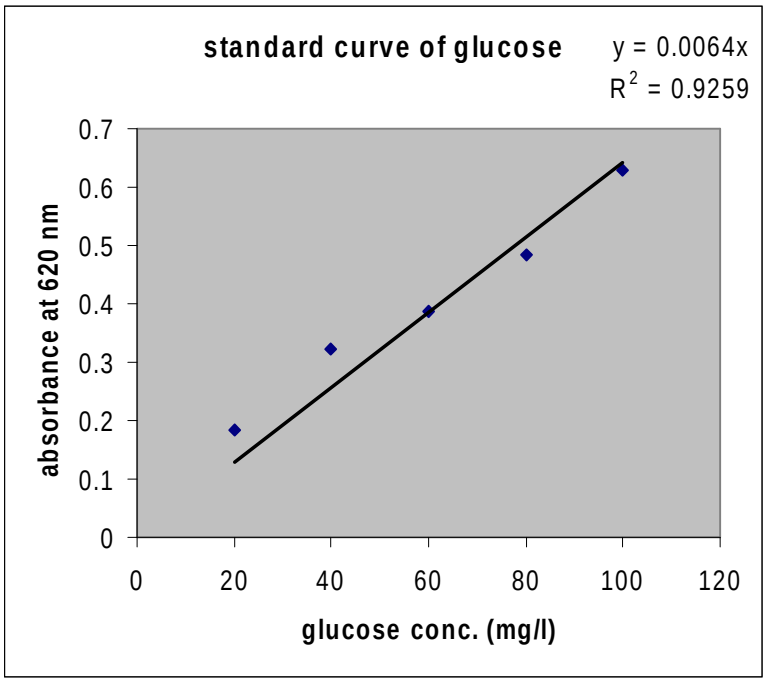
2. NUTRIENT BROTH MEDIUM.

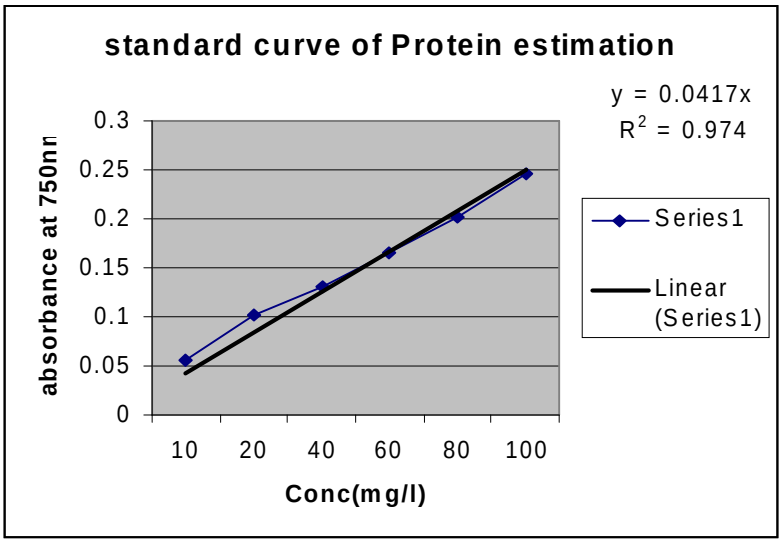
Peptone	5.0
Beef Extract	3.0
Distil Water	1.0 L
pH: 6.6 – 7.0	

Dissolve ingredients in 1.0 L of distil water. Dispense into appropiate glassware & autoclave at 121°c for 15min.

ANNEXURE - II

Annexure 2


standard curve of glucose
y = 0.0064x
R2 = 0.9259
absorbance at 620 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
glucose conc. (mg/l)


standard curve of Protein estimation
y = 0.0417x
R2 = 0.974
absorbance at 750nm
0.3
0.25
0.2
0.15
0.1
0.05
0
10
20
40
60
80
100
Conc(mg/l)
Series1
Linear (Series1)

CONCLUSIONS

Conclusion

1. Fourteen bacterial isolates were screened from industrial wastewater, activated sludge.

2. The isolates were selected from these on the basis of their high flocculating activity. The Maximum Flocculation activity was observed in case of two isolates namely RDL1 and RDL2. These Bacterial strains Were Isolated from Textile dye industry.

3. The isolates were named as (RDL1) and (RDL2) and studied further in terms of time for flocculant production, carbon, nitrogen, pH, cations, stress, static condition, Were the parameters that were optimized in The study To obtain the maximum Flocculant Production.

3. RDL1 and RDL2 both grew maximally on glucose, ammonium sulfate, and peptone as Carbon and Nitrogen sources. Maximum Flocculant production was achieved after 12 hours in case of RDL1, while in case of RDL2 it was achieved after 60 hours.

4. Starvation of carbon, nitrogen decreases flocculation activity in both the isolates. at a pH of 7.5 (slightly alkaline) both the isolates show maximum flocculating activity.

5. Among cations flocculating activity is maximum seen in case of Ca++ in both the isolates.

6. By giving static condition flocculating activity can be decreased in both the isolates.

7. The yield of the bioflocculants after partial purification by ethanol precipitation was higher in case of isolate RDL1. Protein and carbohydrate both residues were present in the bioflocculants. The total carbohydrate was more than that of protein content in both the isolates RDL1 and RDL2

8.The Studies carried out with the isolates RDL1 and RDL2 reveals that they could be Potential Strains. They might have Wider application. However their efficacy

for various industrial treatment process needs to be checked confirmed its future application of these two promising strain.

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