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Improved production, characterization and flocculation properties of poly (γ)-glutamic acid produced from *Bacillus subtilis*

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Abstract

Bacillus subtilis 2063 produced extracellular biopolymer which showed excellent flocculation activity. The biopolymer was confirmed as poly (γ-glutamic acid) (PGA) by using product characterization. HPLC profile showed that molecular weight of PGA was found to be 5.8×10^6 Da. Improved production, Characterization and flocculation properties of PGA produced by Bacillus species were studied. PGA produced by B. *subtilis* was devoid of any polysaccharides. The flocculating activity was markedly stimulated by the addition of cations. The pH of reaction mixture also influenced the flocculating activity. Glycerol and ammonium chloride were found to be most useful carbon and nitrogen sources. An overall 4.24-fold increase in protease production was achieved in the design medium composed with Glycerol and ammonium chloride as a carbon and nitrogen sources as compared with basal media. PGA production increased significantly with optimized medium (21.42 gl^{-1}) when compared with basal medium (5.06 gl^{-1}).

Keywords: Lipase, SDS-PAGE, *Bacillus subtilis*, Chromatography

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Introduction

Flocculation is, in the field of chemistry, a process where colloids come out of suspension in the form of floc or flakes. This common and effective technique normally used in wastewater treatment for removing suspended solids and metal ions (Deng et al. 2003). It may also be used as an alternative of centrifugation and filtration for separation of microbial cells from broth in food and Pharmaceutical and medicine industries (Shih & Van 2001). Flocculants are normally classified into synthetic organic flocculants, synthetic inorganic flocculants, and bioflocculants.

However, the drawbacks of synthetic organic flocculants are less biodegradable and producing carcinogenic monomers such as acrylamide during degradation (Salehizadeh et al. 2000). Quite expectively to save our environmental and health, the development of safe and biodegradable flocculants is an urgent agenda in renowned industry. Bioflocculants are composed of high molecular weight biopolymers such as proteins, glycoproteins, and polysaccharides and they are biodegradable in nature secreted by microorganisms (Suh et al. 1997; Takeda et al. 1992). As a consequence of their safety and eco-friendly nature, bioflocculants have gained much attention among researchers (Li et al. 2003). Bioflocculants are also used in both aerobic and anaerobic treatment process for the formation and settlement of sludge (Houghton & Quarmby 1999) and therefore it can be used as potential and attractive applications in wastewater treatment and other relevant industries. However, drawbacks of bioflocculants are high cost and low flocculating activity and which has limited the feasibility of their practical applications in industries.

PGA is anionic naturally occurring homo-polyamide is water soluble, biodegradable, and non toxic towards humans and the environment. Although there are several microbial PGA producers, only a few are considered industrially useful (Shih & Van 2001). Gram-positive bacteria especially the genus *Bacillus* is considered as an important commercial PGA producer. Keeping in view of the demand, usefulness and cost effectiveness of the bioflocculant in the industry, it is very important to focus on their overall production and that can be done for commercial viability via large-scale fermentation along with cost-effective downstream process (Wu & Ye 2007). Thus, development of influenced medium for production of bioflocculant requires selection of mainly carbon and nitrogen source (Goto & Kunioka 1992; Ko & Gross 1998). It is obvious that process operating condition like optimum media composition plays

a vital role in the improvement of bioflocculant production and have a great influence on the overall process economics, because each microorganism has its own individual physicochemical and nutritional requirements for growth and bioflocculant secretion (Goto & Kunioka 1992; Ko & Gross 1998). Accurate selection of media components influences the activities of microorganisms and improves the production significantly, which is desirable for minimization of processing cost. At industrial level, even small improvement of yield may be beneficial commercially (Reddy et al. 2008).

In this study, experiment has been made on characterization of bioflocculant and its flocculation properties. Then, selection of carbon and nitrogen source for influenced media design was carried out by one-factor-at-a-time method for bioflocculant production from *Bacillus subtilis 2063*.

Materials and Methods

Chemicals and Analysis

All chemicals were analytical grade and all experiments were carried out in triplicate.

Microorganism and seed culture

Bacillus *subtilis* 2063 was procured from National Chemical Laboratory, Pune, India. The microorganism was grown on nutrient agar slants at 37°C at pH 7.4. It was maintained by sub-culturing on nutrient agar slants kept at pH 7.4. For production experiments, the culture was revived by adding a loop full of pure culture into 50 ml of sterile nutrient broth (pH 7.4).

Bioflocculant production

A 2% fresh culture (A550 nm ≈ 0.2) was inoculated in 50 ml complex media of 250 ml Erlenmeyer flask, containing (gl^{-1}); glucose, 25; citric acid, 10; L-glutamic acid, 20; NH_4Cl, 6; K_2HPO4, 1; $MgSO_4$, $7H_2O$, 0.5; $CaCl_2$ $2H_2O$, 0.2; $FeCl_3$ $7H_2O$, 0.03; at pH-7 and incubated at 37°C, with 180 rpm for 96 h (Du et al. 2005).

Purification of bioflocculant

Culture broth was appropriately diluted and cells were separated from broth by centrifugation for 20 min at 10000 rpm and 4ºC. The supernatant was poured in to four volumes of methanol and kept for 12 h at 4ºC. Crude bioflocculant was collected by centrifugation for 30 min at 10000 rpm and 4ºC and dissolved in distilled water and any insoluble impurity was removed by centrifugation. The aqueous solution was desalted by dialysis (Molecular weight cut-off 12 kD) and finally lyophilized to prepare pure material (Goto & Kunioka 1992).

Characterization of Bioflocculant

Amino acid analysis

For amino acid analysis purified bioflocculant was hydrolyzed with 6N HCl at 110 °C for 24 h in a sealed and evacuated tube. Thin-layer chromatography was performed on a cellulose plate using solvent systems of butanol–acetic acid–water (3:1:1, ww^{-1}) and 96% ethanol–water (63:37, ww^{-1}). Amino acids were detected by spraying with 0.2 % ninhydrin in acetone (Shih et al. 2001).

Total sugar content

The total sugar content of the purified bioflocculant was determined by the phenol–sulphuric acid method (Dubois et al. 1956).

Molecular weight determination.

Waters ^{TM} 600 Pump based HPLC system equipped with Waters quaternary pump, Waters manual injector, Waters on-line degasser AF and Waters Refractive index Detector was used. Water empowered software (Version: Empower 2 software Build 2154) was used for data acquisition and mathematical calculations. The number average molecular weight of the bioflocculant was measured by gel permeation chromatography (GPC) using – controller along with Shodex K-800 series column (KF-805). Dextran-polysaccharide standard obtained from waters was used to construct calibration curve from which molecular weight of flocculants were calculated. The eluting contained 0.3 M Na_2SO_4, 0.05% (WV^{-1}) NaN_3 was brought to a pH-4 using glacial acetic acid and flow rate was set 1 mlmin^{-1}.

Flocculating activity of cultural medium

The flocculating activities were measured as per the method of Yokoi et al. (1995) after slight modifications. 5 gL^{-1} of kaolin and 4.5 mM $CaCl_2$ was mixed in distilled water to prepare a suspension. 9.3 ml of the suspension was added to a test tube, and supplemented with various amounts of culture broth. A control experiment using distilled water instead of culture broth was also carried out. The mixture was gently mixed and allowed to stand for 5 min at room temperature (28 - 30°C). The formation of visible flocs was observed. The optical density (OD) of the upper phase was measured on a spectrophotometer (U-2800, Hitachi, Japan) at 550 nm. All assays were conducted in triplicates. Flocculating activity was calculated according to the following equation (Kurane et al. 1986).

Flocculating activity = 1/(OD550)s – 1/(OD550)c
(OD550)s =Optical density of sample at 550 nm,
(OD550)c =Optical density of control at 550 nm

Factors affecting on flocculating activity

PGA concentration, cation concentration and pH are considered to contribute flocculating activity.

To study the effect of PGA concentration on the flocculating activity, the kaolin suspension (5 gL^{-1}) 9.3 mL was supplemented with various amounts of PGA solution to achieve 2.5, 5, 10, 15, 20, 25, 30, 40 or 50 mgl^{-1} of PGA concentration. $CaCl_2$ concentration was adjusted to 4.5 mM. In control experiment distilled water was used instead of PGA solution. The flocculating activities were then measured and calculated. To evaluate the effect of cations on the flocculating activity, flocculation tests were carried out using $CaCl_2$, KCl, $MgCl_2$, $FeCl_3{\cdot}6H_2O$ and $AlCl_3{\cdot}6H_2O$ at various concentrations (0.1, 1.0, 2.5, 5, 10, 15 and 20 mM). PGA concentration in all the cases was 10 mgl^{-1}. Flocculating activities were then measured. To study the effect of pH on the flocculating activity, the reaction mixtures composed of a kaolin suspension, PGA (10 mgl^{-1}) and $CaCl_2$ (5mM), were adjusted to predetermined pH values of 4.0 to 10.0. Flocculating activities were then measured
The precipitates were then resuspended in 10 mM phosphate buffer, pH 7.2 and allow to stand overnight at 4°c. after incubation the enzyme was dialyzed against 40% sucrose solution to get concentrated enzyme free from salt and metal ions.

Optimization of Bioflocculant production

To evaluate effect of media components on bioflocculant production, carbon source and nitrogen source are considered to contribute bioflocculant production from *Bacillus sp.* (Goto & Kunioka 1992; Ko & Gross 1998).

Selection of most suitable carbon, nitrogen and Metal ion from *Bacillus sp*. Were carried out by one variable at a time method. In the production medium, glucose was replaced by different carbon sources viz. Starch, mannitol, glycerol, sorbitol, maltose, lactose sucrose, mannose and fructose. All carbon sources were used at a final concentration of 1% wv^{-1}. Various organic and inorganic nitrogen sources were added to the fermentation medium at a final concentration of 0.5% wv^{-1} (Chauhan & Gupta 2004; Puri et al. 2002).

To study the effect of different nitrogen sources on bioflocculant production, ammonium chloride was replaced with other organic as well as inorganic nitrogen sources such as peptone, soybean meal, casamino acid, gelatine, tryptone, urea, yeast extract, sodium nitrate, ammonium nitrate, ammonium sulphate and potassium nitrate.

Selection of sulphate source was carried out by one variable at a time method where various metal sulphates sources such as magnesium sulphate, ferrous sulphate and calcium sulphate were added to the fermentation medium at a final concentration of 0.5 gl^{-1}. Flocculating activity was determined after 96 h of incubation at 37°C, with 180 rpm.

Citric acid and glutamic acid were considered as precursor substrates for polymer production (Cromwick & Gross 1995; Ko & Gross 1998).so their concentrations were unchanged.

Results and Discussions

Characterization of Bioflocculant

Bioflocculant obtained from *B. subtilis* was characterized by amino acid analysis with thin-layer chromatography. Thin-layer chromatography of the hydrolysate performed on a cellulose thin-layer plate and visualized with 0.2% ninhydrin indicated a single spot with R_f value which is identical as authentic glutamic acid. This finding indicates that bioflocculant produced by *Bacillus subtilis* is PGA. The purified bioflocculant was tested by the phenol–sulphuric acid for polysaccharide content. No sugar was detected in PGA indicating that *B. subtilis* did not produce polysaccharides as by-product. The molecular weight of PGA obtained from *B. subtilis* was determined by Gel permeation chromatography (GPC) and found to be over 5.8×10^6 Da. It is known that the molecular weight of PGA varies from 10^5 to 10^6 Da depending upon the species and the cultivation conditions used for its production (Shih & Van 2001). Under the conditions used in this study, the molecular weight of PGA produced by *B. subtilis* is comparable to the results obtained so far with other species.

Factors affecting on flocculating activity

The effect of PGA concentration on the flocculating activity was shown in Figure 1. The result showed that flocculating activity increased with bioflocculant concentration up to 10 mg/L, which showed the highest (18.498 ± 0.893) activity and decreased thereafter. This finding was found as similar with many published reports (Wu & Ye 2007; Yokoi et al. 1995). There are several reports which suggested that flocculating activity was stimulated by cations. Cations influenced flocculating activity by neutralizing and

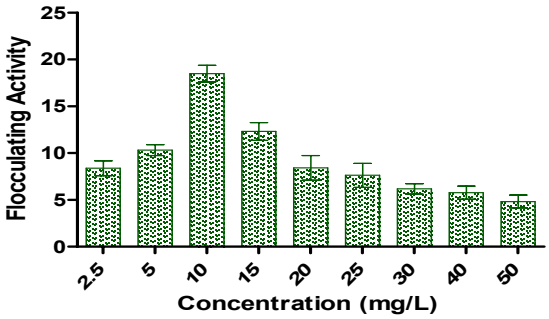

Figure 1: Effect of PGA concentration on flocculating activity

stabilizing the negative charge of functional groups by forming bridging between particles. The stimulating effect of cations is depending on both the concentration and charge of the ions (Wu & Ye 2007; Yokoi et al. 1995). The effect of cations and their concentrations on flocculating activity of PGA was shown in Figure 2.

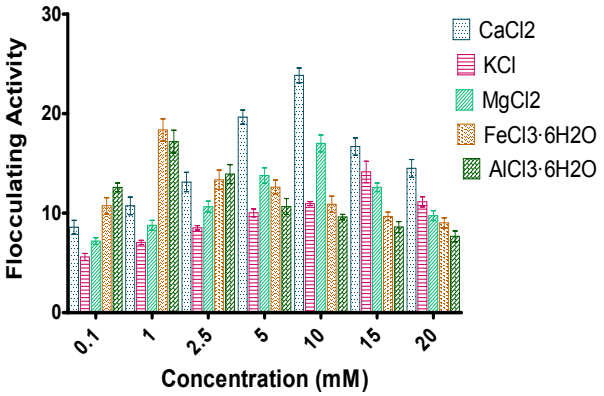

Figure 2: Effect of cation at various concentrations on flocculating activity of PGA

Optimum concentration of Ca^{2+}, K^{1+}, Mg^{+2}, Fe^{3+} and Al^{3+} for flocculating activity of PGA were 10, 15, 10, 1 and 1.0 mM respectively. Maximum flocculating activity (23.836 ± 0.744) was obtained with Ca^{2+} at 10 mM. Flocculating activity decreased when these cations were used above their optimum concentrations. Monovalent cations like K+ also increased flocculating activity, but the effect was very less as compared to divalent cations. Neutralization of charge plays an important role in flocculation. The charge bridging between the bioflocculants increases the floc density, floc size and the floc resistance to shear. Ca^{2+} and Mg^{2+} destabilize the negatively charged kaolin particle by neutralizing and bridging and support flocculation. Monovalent cations reduce the strength of the bonds and cause a loose structure of flocs, thus resulting in a decrease in flocculating activity (Gong et al. 2008; Wu & Ye 2007).

The pH of reaction mixtures is a key factor influencing flocculating activity. As shown in Figure 3, the flocculating activity clearly increased near neutral pH. Maximum flocculating activity (23.77 ±

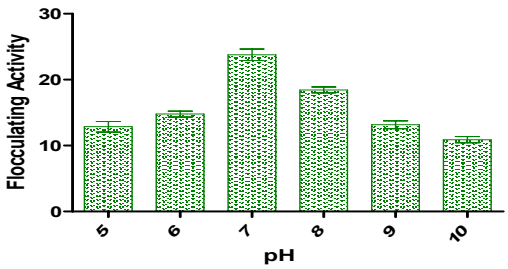

Figure 3: Effect of pH on flocculating activity of PGA

0.878) was obtained with the addition of Ca^{2+}(10 mM) at pH 7. The preferable pH for flocculation obtained with PGA is similar to that of flocculant produced by *B. subtilis* DYU500 (Wu & Ye 2007), but different from that for bioflocculant produced by *Bacillus* sp. PY-90 as the optimal pH was approximately 4.0 in the presence of Ca^{2+} (Yokoi et al. 1995). PGA produced from *B. Licheniformis* also gave a maximum flocculating activity with Ca^{2+} at neutral pH. These results suggest that flocculation is due to change in charge density. Addition of cations at particular pH may result in neutralization of zeta potential (Shih et al. 2001).

Optimization of bioflocculant production from Bacillus subtilis

Glycerol, ammonium chloride and magnesium sulphate are considered as most useful carbon nitrogen and metal ion source on bioflocculant production by one variable at a time method and shown in Figure 4-6. It was also observed that bioflocculant production improves in the presence of simple carbon and inorganic nitrogen sources, viz. glucose and ammonium chloride. There are published reports of improvement bioflocculant production for other *Bacillus species* in the presence of simple carbon and inorganic nitrogen sources (Bajaj et al. 2009; Du et al. 2005; Goto & Kunioka 1992; Ko & Gross 1998; Shih et al. 2002). Repressive effect on flocculating activity was observed when other carbon sources were used. Catabolite repression may be the most likely reason for this lagging effect (Kumar et al. 1999; Priest 1977). It was previously established that a catabolite control protein (CcpA) was responsible for this regulatory mechanisms which transduced signal for the repression in bioflocculant synthesis (Tobisch et al. 1999)..

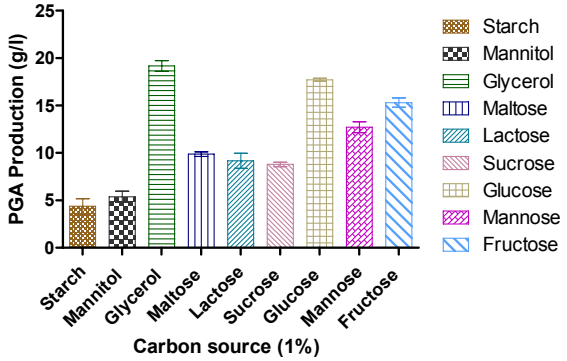

Figure 4: Effect of different carbon sources on production of PGA after 96 h incubation.

Nitrogen sources other than ammonium chloride showed a relative decrease in bioflocculant production. This can be explained by the mechanism of feedback inhibition (Malathi & Chakraborty 1991). Magnesium ion was found to be more effective in bioflocculant production than other metal ions. The depletion in magnesium ion results in decreased rate of enzyme production that ultimately affects the mechanism of bioflocculant synthesis (Hanlon et al. 1982).

There are several published reports on production and application of bioflocculants such as *Rhodococcus erythropolis S-1* (Kurane et al. 1986), and *Bacillus* sp. *DP-152* (Suh et al. 1997) produced protein flocculants. *B. licheniformis* (Shih et al. 2001), *B. subtilis DYU1* (Wu & Ye 2007), *B. subtilis* (Mahmoud 2006) produced polyamide flocculants. *Alcaligenes cupidus KT201* (Toeda & Kurane 1991) and *B. mucilaginosus (Lian et al. 2008)* produced polysaccharide flocculants. Among these bio-flocculants, PGA, a polyamide flocculants is considered as best bioflocculant because of its high yield, high flocculating activity and ability to flocculate wide range of organic and inorganic compound (Shih & Van 2001).

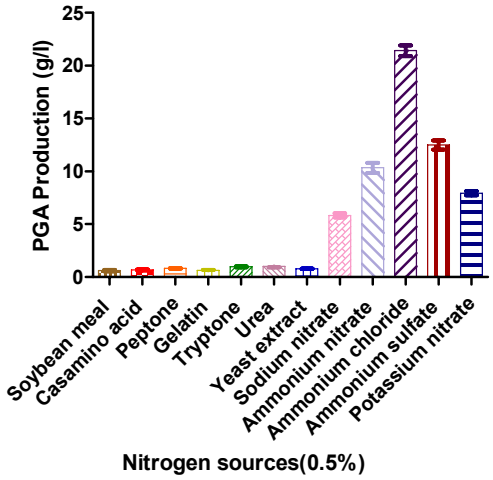

Figure 5: Effect of different nitrogen sources on production of PGA after 96 h incubation.

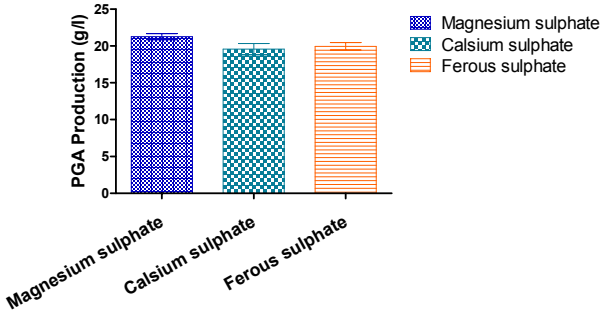

Figure 6: Effect of different metal ion on production of PGA after 96 h incubation

A 4.24-fold higher PGA production by *Bacillus subtilis 2063* was achieved under design media compared to basal media. *B. subtilis* produced 21.42 gl^{-1} of PGA after 96 h of fermentation (Figure 7). The PGA yield in the present study is quite comparable with the reports till date and proper optimization of media and physical components can also improve PGA production. The maximum PGA production reported by using most widely strain *B. lichenifomis*

ATCC 9945A and *B. subtilis* IFO 3335 is 23 and 20 gl^{-1} (Cromwick et al. 1996; Kunioka & Goto 1994). PGA production reported from *B. lichenifomis* CCRC 12826 and *B. subtilis* are 19.62 and 15.6 g/L (Ashiuchi et al. 2001; Shih et al. 2002). PGA yield obtained by using a mutated strain of *Bacillus licheniformis* CICC10099 was 16.9 gl^{-1} (Suo et al. 2007).

Conclusion

Due to efficiency and economic concern, the present study was focused on characterization and selection of media components for maximal bioflocculant production through microbial fermentation. The PGA produced by *Bacillus subtilis 2063* had a molecular weight of 5.8×10^6 Da, and a good flocculating activity that can be mostly promoted by the addition of bivalent and trivalent cations. PGA can be a potential replacement of conventional synthetic flocculants in wastewater treatment and downstream processing of food and pharmaceutical and medicine industries due to its safety and eco-friendly character. Further experiments on this bioflocculant towards optimization of media and physicochemical parameters are currently under way.

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