**OPTIMIZATION OF THE PHYSICO-CHEMICAL PARAMETERS FOR THE PRODUCTION OF BIOFLOCCULANT BY BACILLUS SPECIES**

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**ABSTRACT**

Several serious problems are associated with the use of conventional synthetic flocculants, especially alum, as coagulants in waste water treatment. *Bacillus* sp. are some of the organisms capable of producing exopolysaccaride bioflocculants. Compared with conventional synthetic flocculants, bioflocculants have special advantages such as safety, strong effect, biodegradable and harmless to humans and the environment. Twenty-five bacterial isolates were obtained from soil samples and were screened for their ability to flocculate kaolin clay suspension. Five bacterial isolates (*Bacillus firmus*, *Bacillus mucilaginosus*, *Bacillus subtilis*, *Bacillus coagulans* and *Bacillus licheniformis*) were selected for further studies based on their flocculating potentialities. Glucose supported the maximum bioflocculant production by all the bacillus strains, ranging from 17.0% to 88.1%. Urea was the best nitrogen source for flocculation. Ca2+ was the best cation with a flocculation rate of 88.1%. A temperature and pH of 350C and 7 respectively were both optimal, rates ranged from 23.2% - 81.9% and 19% - 80% for temperature and pH respectively. Agitation (120rpm) better supported flocculation than static condition, flocculation rates ranged from 28.6% - 88.1%. Chemical analyses of the purified bioflocculants revealed that the major component of the bioflocculants was polysaccharide which ranged from 0.27% - 0.68%. The dry weight of the bioflocculants produced by the bacillus strains follows a linear increase from *B. firmus*, with a value of 5.2g/l through *B. mucilaginosus* with the highest value of 8.7g/l. Optimization of the physico-chemical parameters had tremendous effects on bioflocculant production and *Bacillus mucilaginosus* gave the best flocculating activity, at probability level p≥0.05. This study shows a useful way of harnessing the flocculating efficacy of *Bacillus* strains, hence, their recommendation, most especially *Bacillus mucilaginosus*.

**Keywords: Exopolysaccharide, synthetic flocculants, kaolin clay, alum, biodegradable**

**INTRODUCTION**

In microbiology, the term bacillus means any rod-shaped microbe. *Bacillus* sp. are largely straight Gram-positive rods occurring in chains which grow aerobically and form heat-resistant spores. They belong to the genus *Bacillus.* The Gram-positive property of strains is variable. The spores are ubiquitous and are extremely common in dust so that a large proportion of bacteria-contaminating cultures belong to this group. These organisms exist as saprophytes in soil, water, air and in vegetation e.g. *B. mycoides* and *B. Subtilis*, *B. anthracis,* the causative organism of anthrax in man and animals, is the only pathogen of the group, though very occasionally , species as *B. subtilis* have been isolated from the tissues in terminal disease (Cruickshank *et al*., 1969).

*Bacillus* sp. are of tremendous industrial importance. Many of them are able to secrete large quantities of enzymes. *B. amyloliquefaciens* is a species of *Bacillus* that is the source of a natural antibiotic protein; barnase (a ribonuclease), alpha amylase used in starch hydrolysis, the protease; subtilisin, used with detergents, and the Bam H1 restriction enzyme used in DNA research (Graumann, 2007).

*Bacillus* sp. are also of clinical importance. Two *Bacillus* sp. are considered medically significant: *B. anthracis*, which causes anthrax, and *B. cereus*, which causes a food-borne illness similar to that of *Staphylococcus* (Ryan, 2004). A third species, *B. thuringiensis*, is an important insect pathogen, and is sometimes used to control insect pests. *B. subtilis* is an important model organism. It is also a notable food spoiler, causing ropiness in bread and related food. *B. coagulans* is also important in food spoilage.

Historically, the term “coagulation” and “flocculation” have been used indiscriminately to describe the process of removal of turbidity from water. There is however a clear distinction between the two terms. The term “coagulation” comes from the Latin word coagulare, meaning to drive together (Faust and Aly, 1998; Nester *et al*., 2001).

The chemical coagulation of turbid water or naturally coloured surface water involves the interaction of particulates and/or colloids with a destabilizing agent. The essential purpose of coagulation is to aggregate these particles into larger sizes that will settle quickly within an hour or two and/or will be filtered by sand or other media. This aggregation process is also called destabilization of colloidal systems. Colloids are characterized by their size and by the mechanism by which they are stabilized in water (Nester *et al*., 2001).

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 thereby hasten the gravitational settling of suspended solids in solution. These small flocs can be built up into larger aggregates by flocculation, with the larger particles formed in this waygiving higher rate of sedimentation (Gutcho *et al*., 1997).

Flocculants are substances having a synthetic or natural origin that are used as sedimentation aids to bring about the solid-liquid separations by the process of flocculation in industrial plants. The use of flocculants is necessary because suspended solids in aqueous solution exhibit Brownian movement which keep them in constant motion and inhibit settling; the purpose of the flocculants is to neutralize the like charges in suspension by coagulating and flocculating them into large size. The larger the particle size, the faster the settling rate. Microbes, especially bacteria have shorter generation times, are versatile and can produce extra cellular polymeric materials which can flocculate; and those which are obtained from natural sources have been termed as ‘bioflocculants’(Lachhwani, 2005).

Bioflocculant, a special macromolecule secreted by microorganisms, induces solid particles, bacteria, cells and colloidal particles in a liquid suspension to flocculate and sediment. So far, microorganisms including algae, bacteria, actinomyces and fungi have been reported to produce bioflocculants. Bioflocculants (microbial flocculants) are polymers produced by microorganisms during their growth (Kurane *et al*., 1994).

In general, flocculants are divided into chemically synthesised flocculants (organic and inorganic flocculants) and natural flocculants (chitosan, algin and microbial flocculants) (Suh *et al*., 1997).

Flocculants are generally classified into three major groups: inorganic flocculants such, as aluminum sulphate and polyaluminum chloride; organic synthetic polymeric flocculants, such as polyacrylamide derivatives and polyethylene amine; and naturally occurring flocculants, such as chitosan and sodium alginate, and microbial flocculants (Kurane *et al*., 1994).

Inorganic and organic synthetic polymer flocculants are frequently used in water and wastewater treatment because they are economical and highly effective. However, their use often gives rise to environmental and health problems in that some of them are not readily biodegradable and some of their degraded monomers, such as acrylamide, are neurotoxic and even strong human carcinogens. Residual alum concentration in treated water can also impose health problems apart from the production of high amount of sludge (Letterman and Driscoll, 1988). There is also a problem of reaction of alum with natural alkalinity present in water leading to a reduction of pH and a low efficiency in coagulation of cold waters (Degremont, 1989; Ndabigengesere and Narasiah, 1998). Thus, the development of safe biodegradable flocculants that will minimize environmental and health risks is urgently required (Shih *et al*., 2001).

Flocculants have been widely used in a variety of industrial processes such as wastewater treatment, food and fermentation industries, drinking water purification and industrial downstream processes (Wu and Ye, 2007).

Several serious problems are associated with the use of conventional synthetic flocculants, especially alum, as coagulants in waste water treatment. These problems include neuro toxicity and Alzheimer’s disease which is characterized by loss of memory (amnesia) and speech loss (Yokoi *et al.*, 1995). Hence, this research work was carried out with the aim of addressing these problems with a health-friendly, yet economically effective alternative from *Bacillus* sp.

**MATERIALS AND METHODS**

Soil samples were randomly collected for use at different areas within the University of Ibadan. The moist soil samples were got from the vegetation directly opposite the department of Agricultural Extension and Rural Development, Abdul-Salam Abubakar Hall, Department of Microbiology and Obafemi Awolowo Hall. The soil samples were collected into clean, pre-sterilized miniature plastic containers, for onward transportation to the laboratory.

A selective medium, Tryptone Soy Agar (TSA), was employed for the isolation of the *Bacillus* sp. by serial dilution. Subsequent sub-culturing was done at 3-weeks interval to obtain axenic colonies. The probable identities of the pure isolates were determined using Bergey’s Manual of Systematic Bacteriology (Peter *et al.,* 1986).

**Characterization of *Bacillus* sp.**

The bacterial isolates were characterized, for further identification, using standard biochemical tests, cellular and colonial morphology. The bacterial isolates were subjected to biochemical tests with reference to Olutiola *et al.* (2000). The isolates’ colonial morphology was determined based on colony surface, colour, shape, elevation and other optical characterization. Stained bacterial smears were prepared and then observed under a light microscope (x 100) to determine their cellular morphology. The bacterial isolates were examined for colonial morphology with reference to Willey *et al*. (2008).

**Bioflocculant Production**

The composition of the Bioflocculant Production Broth medium (BPB) for screening was as follows: 10g glucose, 2g KH2PO4, 5g K2HPO4, 0.2g MgSO4·7H2O, 0.1g NaCl, 0.5g CaCO3, and 0.5g yeast extract dissolved in 1 litre deionized water with the initial pH adjusted to 7.0. After sterilization at 1210C for 20mins and inoculation of the medium, the bacterium was cultured on a rotary shaker at 150 rpm and 370C for 2 days. Kaolin suspensions at a concentration of 5,000mg/l were then used to evaluate the flocculating capability of a series of the culture broths. From these results, a bacterium with good flocculating capability was selected and cultivated in 100ml medium in a 250 ml flask on a shaker at 150 rpm and 30C for 84 h. Medium samples were taken at appropriate time intervals to determine the pH, OD550(optical density at 550 nm) and flocculation properties (Zheng *et al*., 2008).

**Screening for Bioflocculant Synthesis**

The screening for bioflocculant production was carried out by kaolin assay (Suh *et al*., 1997). This assay is a measure of the flocculating capability of the bioflocculant. A 0.5g of kaolin clay (or 10g litre-1 CaCl2) was suspended in 100ml of deionized water and 2ml of the culture broth containing the bioflocculant was added to the kaolin suspension (Kurane *et al*., 1994). The mixture was stirred vigorously for 20s and left without shaking for 10min. The absorbance of the supernatant and blank control without flocculant was measured at 550nm (as OD550 and ODblank, respectively) with a spectrophotometer. The flocculating rate was defined and calculated as follows:

Flocculating rate (%) = (ODblank – OD550)/ ODblank x 100,

OD550 – Optical density of the sample at a wavelength of 550nm

ODblank – Optical density of the blank control at a wavelength of 550nm.

**Effect of Physico-chemical Parameters on Bioflocculant Production**

The following physiological characterization was carried out on the screened *Bacillus* strains as described by Lachhwani (2005); effect of carbon sources, organic/inorganic nitrogen sources, cations, pH, temperature, static and agitation and Stress Conditions on the selected isolates.

**Purification of the Bioflocculant**

The extracellular polysaccharide was puriﬁed from the culture broth by the method of Seinosuke *et al.* (1981) with some modiﬁcations.

**Chemical Analyses of the Bioflocculants**

Total sugar content of the purified bioflocculant was determined by the phenol–sulphuric acid method using glucose as the standard solution as described by Chaplin and Kennedy (1986).

Total protein content was measured by the Lowry *et al.,* (1951) method using bovine serum albumin as the standard solution.

**RESULTS**

A total of 25 bacteria were isolated from different soil samples. All the isolates were screened for their ability to produce bioflocculant. Twelve *Bacillus* strains were selected based on their ability to produce bioflocculant as shown in Fig. 1. The best 5 *Bacillus* strains (*Bacillus firmus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus coagulans* and *Bacillus mucilaginosus*) were selected based on their satisfactory flocculating potentiality exceeding 50%. They were chosen for further studies. The *Bacillus* strains were determined to be Gram-positive rods, encapsulated endospore formers and positive to catalase test, positive to methyl red and nitrate reduction tests. While most of the isolates hydrolysed starch, xylose, glucose, maltose, sucrose and arabinose the same couldn’t be said about gelatin. Various morphological and biochemical tests carried out on the *Bacillus* strains were used to give their probable identities as shown in Table 1. 

**Table 1: Biochemical tests for the Identification of the Bacterial Isolates**

|  |  |
| --- | --- |
| Biochemical tests |  Isolates’ codes |
|  | **SS4b** | **SS7** | **SS4a** | **SS4g** | **SS2** |
| Gram’s stain | +ve | +ve | +ve | +ve | +ve |
| Shape | Rod | Rod | Rod | Rod | Rod |
| Catalase | +ve | +ve | +ve | +ve | +ve |
| Spore | +ve | +ve | +ve | +ve | +ve |
| Motility | +ve | +ve | +ve | +ve | +ve |
| Starch | +ve | +ve | +ve | +ve | +ve |
| Maltose | +ve | +ve | +ve | +ve | +ve |
| Glucose | +ve | +ve | +ve | +ve | +ve |
| Xylose | +ve | +ve | +ve | +ve | +ve |
| Arabinose | +ve | +ve | +ve | +ve | +ve |
| Sucrose | +ve | +ve | +ve | +ve | +ve |
| Gelatin | +ve | +ve | -ve | +ve | +ve |
| Nitrate | +ve | +ve | +ve | +ve | +ve |
| Urease | -ve | +ve | -ve | -ve | +ve |
| Citrate | +ve | +ve | +ve | -ve | +ve |
| Vogres-Proskauer | +ve | +ve | +ve | +ve | -ve |
| Methyl red | +ve | +ve | +ve | +ve | +ve |
| Indole | -ve | -ve | -ve | -ve | +ve |
| Oxidase | -ve | +ve | -ve | -ve | -ve |
| H2S | -ve | +ve | -ve | -ve | -ve |
| Capsule | +ve | +ve | +ve | +ve | +ve |
| Probable identity | *Bacillus coagulans* | *Bacillus subtilis* | *Bacillus licheniformis* | *Bacillus mucilaginosus* | *Bacillus firmus* |

**DISCUSSION**

Most bioflocculants are produced by microorganisms during their growth periods (Kwon *et al.*, 1996; Nakata and Kurane 1999; Shih *et al.,* 2001). Bacteria can utilize the nutrients in the culture medium to synthesize high molecular weight polymers internally within the cell under the action of specific enzymes, and these polymers can be excreted and exist in the medium or on the surface of the bacteria as capsule. Therefore, the action of bacteria converts the simple substances in their environment into complex polymers that can be used as flocculant (Deng *et al.*, 2003). The correlation between cell growth and secretion of extracellular biopolymeric flocculants was originally reported by McKinney (1956).

According to the findings of this study, environmental and nutritional parameters played important roles in bioflocculant production and flocculating activity. Among the various parameters tested, glucose was the most effective carbon source, though sucrose also greatly induced the production of bioflocculants. Glucose gave the best yield probably because glucose can be easily metabolized by the *Bacillus* strains (Ikram *et al.*, 2006).

Urea was the best organic nitrogen source while ammonium sulphate was the best inorganic nitrogen source utilized for the production of bioflocculant by the *Bacillus* strains.

Calcium chloride, magnesium sulphate and manganese sulphate all induced bioflocculant production but with calcium chloride being the best inducer. Cations stimulate flocculating activity by neutralizing and stabilizing the residual negative charge of functional and by forming bridges between particles (Buthelezi, 2009). Calcium may create denser sludge flocs through a decrease in bound water associated with the flocs (Foster, 1983).

Among the five pH levels tested, pH value of 7.0 was the most suitable as this might be due to the *Bacillus* strains’ requirement of near alkaline pH for bioflocculant production.

The optimal temperature for bioflocculant production was attained at 35oC and as the temperature increased, there was a gradual increase in the production of the bioflocculant but at 40oC, production began to reduce. Reduction in bioflocculant production may be due to the fact that high temperature can change membrane composition and can cause inhibition of bacterial growth, likewise nutritional deficiencies.

As agitation (120 rpm) increased the flocculation rates, incubation without shaking and nutritional stress decreased them. All these were achieved greatly at the 24th hr of incubation.

These results are in agreement with the findings of Lachhwani (2005), but with the exception of peptone as the best nitrogen source, which is at variance with that of this study. Also, the bacterial bioflocculants produced were composed of carbohydrates and proteins, though the proteins were in minute quantities and this gives credence to the work of Higgins (1995). Higgins posited that bacterial bioflocculants are generally low in proteins and that extracellular proteins in flocs are associated with improvements in settling and dewatering properties

The bioflocculants production was found to be time dependent. The maximum yield was achieved by *B. mucilaginosus* and it was 8.7 g/l when 1 % and 1.5% of glucose and urea were used as carbon source and nitrogen source, respectively, in thebasal medium.

The bioflocculants produced by these *Bacillus* strains were capable of flocculating kaolin clay suspension. However, flocculating rates declined after a certain period this may be because an optimum amount of flocculants in the suspension causes a larger amount of kaolin particles to aggregate and settle. According to Chan and Chiang (1995), the amount exceeding the optimum concentration of flocculants is known to cause the aggregated particles to re-disperse and would also disturb particle settling. This behaviour could also be caused by an increase in the repulsive energy between the flocculants and kaolin in solution, which causes hindrance in floc formation (Mishra *et al.*, 2004).

In order to record high flocculating activity by the *Bacillus* strains, it was essential to optimize the physico-chemical parameters.

**CONCLUSION**

In conclusion, bioflocculant synthesis by *Bacillus* sp. has a satisfactory level of flocculating activity and could be improved by optimizing the physico-chemical parameters which include the basal medium composition, temperature, pH and incubation in a rotary shaker (agitation).

*B. mucilaginosus* was discovered to be the most promising among the selected five isolated bacteria from the same genus, hence, its possibility to have a wide industrial application.

**RECOMMENDATION**

Further studies could be carried out on how to genetically modify *B. mucilaginosus* for industrial use, basically, in biotechnology, for more desirable potent results.

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