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Full Length Research Paper

Studies on bioflocculant production by *Methylobacterium* sp. Obi isolated from a freshwater environment in South Africa

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A bioflocculant-producing bacterium isolated from river water was identified as *Methylobacterium* sp. Obi by 16S ribosomal ribonucleic acid (rRNA) gene sequencing. The nucleotide sequence was deposited in the Genbank with accession number HQ537130. The bioflocculant secreted by the isolate showed a peak flocculating activity of 72% in 1 g/L kaolin suspension supplemented with $CaCl_2$. The bioflocculant yield was maximal when glucose and peptone were used as carbon and nitrogen sources respectively, at an optimum pH of 7. Calcium was the most effective cation in stimulating bioflocculating activity. Chemical analyses of the purified bioflocculant revealed it to be a polysaccharide.

Key words: Freshwater, *Methylobacterium* sp. Obi, bioflocculant, flocculating activity, polysaccharides.

INTRODUCTION

Bioflocculants are compounds produced by microorganisms, which promote flocculation by causing colloids and other suspended particles in liquid to aggregate or form a floc. They are nontoxic, biodegradable and harmless to both humans and the environment (ABD-EL-Haleem et al., 2008). Louis Pasteur was the first person to discover the presence of flocculants in a microorganism system (Gong et al., 2008). Further studies established the existence of a correlation between the accumulation of extracellular bioflocculants and cell aggregation (Jie et al., 2006).

The use of bioflocculants is encouraged due to the fact that most chemical flocculants that are in use showed side effects to human health and also contribute to environmental pollution. For instance, although polyacrylamide is a frequently used chemical flocculant, there is evidence that the acrylamide monomer is not only neurotoxic but also non-degradable (Zheng et al., 2008).

Bioflocculants have a lot of characteristics that make them a perfect substitute for chemical flocculants, such as safety, biodegradability and the absence of secondary pollution (Li et al., 2008), and as such, can be safely applied in drinking water, wastewater purification and downstream processes in fermentation industries. Recently, bioflocculants have attracted research interest; hence more focus is being directed to the application of microbial bioflocculants in various fields (Gong et al., 2008).

Even though bioflocculants are potentially suitable for application in various industries, there is a need to improve productivity by screening for new microorganisms which can produce flocculants with high flocculating activity (Feng and Xu, 2008). Most of these bioflocculants are mainly composed of polysaccharides, proteins, nucleic acid and some other macromolecular compounds and are mainly used in wastewater to remove the dyes, inorganic solid suspensions such as bentonite, activated carbon, kaolin, calcium hydroxide, aluminium oxide, humic acid and other suspensions

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Abbreviations: EBFs, Extracellular biopolymeric flocculants; **PCR,** polymerase chain reaction; **rRNA,** ribosomal ribonucleic acid.

(Deng et al., 2005).

Over the past decades, a number of microorganisms, including algae, bacteria, actinomycetes and fungi, have been reported to produce bioflocculants (Takagi and Kadowaki, 1985; Zhang et al., 1999; Huang et al., 2005), but majority of microbial bioflocculants have come from *Bacillus* species (Salehizadeh and Shojaosadati, 2001; Deng et al., 2003; Kwon et al., 1996; Suh et al., 1997).

He et al. (2004) investigated the production of a polygalacturonic acid bioflocculant REA-11 from a newly isolated *Corynebacterium glutamicum* CCTCC M201005 strain while Kurane et al. (1986) also reported that *Nocardia restricta*, *Nocardia calcarea* and *Nocardia rhodnii* could produce biopolymer flocculants. In this paper, we assess the bioflocculant production potential of *Methylobacterium* sp. Obi, a freshwater bacterium isolated from the Tyume River, in South Africa.

MATERIALS AND METHODS

Source of bacteria and culture media

Over 200 freshwater bacteria isolated from the Tyume River were screened for bioflocculant production using a cultivation medium described by Zhang et al. (2008), and composed of the following: 10 g glucose, 1 g peptone, 0.3 g $MgSO_4.7H_2O$, 5 g K_2HPO_4, 2 g KH_2PO_4 in a litre of distilled water. The initial pH was adjusted to 7.0 with either NaOH (0.1 M) or HCl (0.1 M).

Screening for flocculant producing microorganism

Each bacterial isolate was inoculated into 5 ml of sterile growth medium contained in a McCartney bottle and incubated at 25°C with shaking (120 rpm) for 7 days. The fermentation broth was centrifuged at 4000 × g for 30 min at 4°C to sediment the cells. The cell free culture supernatant was used assayed for flocculating activity.

Determination of flocculating activity

Using a suspension of kaolin clay as test material, flocculating activity was measured according to the method described by Kurane et al. (1994) and Zhang et al. (2008). Three millilitres of 1% (w/v) $CaCl_2$ and 2.0 ml of bioflocculant were added into 100 ml of kaolin suspension (4.0 g/l) in a 250 ml conical flask, the mixture was vigorously stirred, poured into 100 ml of measuring cylinder and allowed to stand for 5 minutes at room temperature. The optical density (OD) of the clarifying solution was measured with a spectrophotometer at 550 nm. A control experiment was prepared in the same way but the bioflocculant was replaced with distilled water. The flocculating rate (FR) was determined by using the formula:

$$FR = \{(A - B)/A\} * 100$$

Where, A and B are optical densities of the control and samples respectively at 550 nm.

Factors affecting bioflocculant production and flocculating activity

The assessment of the effects of different carbon and nitrogen sources on bioflocculant production by the test bacterium was done according to the method described by Lachhwani (2005). Carbon source candidates included glucose, sucrose, fructose, lactose and starch, while the nitrogen source candidates were peptone, ammonium sulphate, ammonium chloride (inorganic nitrogen sources) and urea (organic nitrogen sources). The effect of salt ions on flocculating activity was investigated by using the following electrolyte solutions as sources of cations: calcium chloride, ferric chloride, magnesium chloride, ferrous sulphate, potassium chloride and the flocculating activity measured as previously described. The effect of pH on flocculating activity was assessed by adjusting the pH of kaolin suspension using HCl or NaOH prior to measuring flocculating activity. The pH values ranged between 3, 4, 5, 6, 7, 8 and 9.

Time course assay for bioflocculant production

The composition of the medium for bioflocculant production was prepared according to the method described by Zhang et al. (2007) by dissolving the following in a liter of deionized water: 10 g of glucose, 1.0 g of peptone, 0.3 g of $MgSO_4 \cdot 7 H_2O$, 5 g of K_2HPO_4 and 2 g of KH_2PO_4. The pH was adjusted to 6.5 with either NaOH or HCl. The selected strain was pre-cultured in 50 ml medium contained in 250 ml flasks on the rotary shaker (120 rpm) at 25°C for inoculation preparation. After 16 h of cultivation, 1% (v/v) of culture broth was used as seed culture to inoculate 400 ml of medium in 1000 ml flasks. Batch fermentation was carried out under the same cultivation conditions as those for pre-cultivation. Medium samples were withdrawn at appropriate time intervals and monitored for pH, cell growth and flocculating activity. Five milliliters of culture broth was centrifuged at 8000 x g for 15 min, and the cell free supernatant was used as the test bioflocculant to determine the flocculating activity.

Chemical analysis of the bioflocculant

Total carbohydrate content was determined by the Phenol Sulphuric acid method with glucose used as a standard solution (Chaplin and Kennedy, 1994). The protein content was measured with Folin-Lowry method as described by Lachhwani (2005) with bovine serum albumin (BSA) as the standard.

Identification of the bioflocculant-producing bacterium

The bacterium was identified by 16S rRNA gene sequencing. For this purpose, DNA was first extracted using boiling method whereby 2-3 pure colonies of the bacterium were suspended in 70 µl of sterile double distilled water in a 1.5 ml eppendorf and boiled in a heating block at 100ºC for 10 min, allowed to cool for 5 min and thereafter centrifuged at 3000 rpm for 5 min to pellet cell debris. The supernatant was transferred to a clean tube and stored at 4ºC. This serves as the template in the PCR assay.

PCR was carried out in 50 µl reaction volume containing 2 mM $MgCl_2$, 2 U Supertherm Taq polymerase, 150 mM of each dNTP, 0.5 mM of each primer (F1: 59-AGAGTTTGATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTTACGACTT-39) and 2 ml template DNA. Primer F1 and R5 binds to base positions 7-26 and 1496-1476 of the 16S rRNA gene of *Streptomyces ambofaciens* ATCC 23877, respectively (Cook and Meyers, 2003). The primers in this study were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used was an initial denaturation (96ºC for 2 min), 30 cycles of denaturation (96ºC for 45 s), annealing (56ºC for 30 s) and extension (72ºC for 2 min), and a final extension (72°C for 5 min). Gel electrophoresis of PCR products were conducted on 1% agarose gels to confirm that a

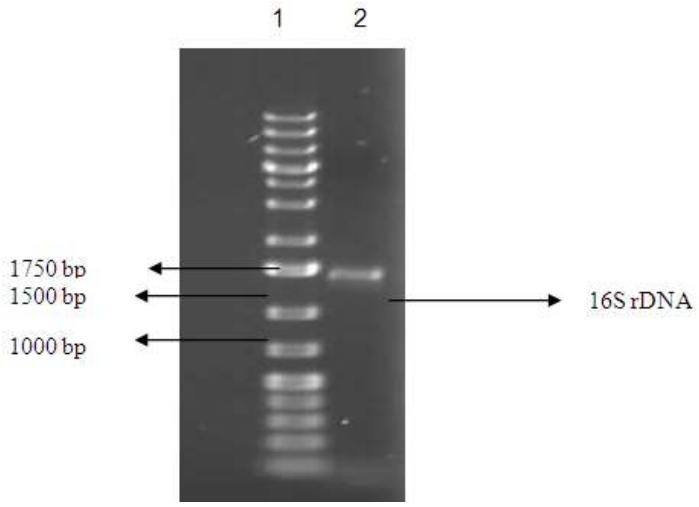

Figure 1. PCR product agarose gel (1%) electrophoresis. Lane 1: DNA markers and Lane 2: PCR product.

fragment of the correct size had been amplified.

Statistical analysis

The results obtained on factors affecting bioflocculant production, flocculating activity and time course for bioflocculant production were analyzed with Statistical Package for Social Scientists (SPSS version 17). A significance level of $p \leq 0.05$ was used. The mean values obtained were subsequently used to chart graphs using Microsoft Office Excel 2007.

RESULTS AND DISCUSSION

Screening and identification of bioflocculant-producing strain

Over 200 freshwater bacteria isolated from the Tyume River in the Eastern Cape Province of South Africa were screened for bioflocculant-producing capabilities. Among these was our tested bacterium which showed a flocculating activity of 72%. PCR amplification of the 16S rRNA gene of the bacterium produced a PCR product of approximately 1.5 kb size (Figure 1). BLAST (Basic Local Alignment Search Tool) analyses of the nucleotide sequence of the amplified product showed a 98% similarity to *Methylobacterium* sp. 440 and the sequence was submitted in GenBank as *Methylobacterium* sp. Obi with accession number of HQ537130. The morphological characteristics of the bacterium revealed the colony to be reddish in colour, round, flat, smooth, and glistening with a colony size of approximately 2 mm in diameter.

Methylobacterium genus is strictly aerobic, Gram-negative and rod-shaped facultative methylotrophs, which use methanol and other reduced mono-carbon compounds via the serine pathway (Green, 1992).

Methylobacteria are classified as α-Proteobacteria and appear as pink to red in colour due to the synthesis of carotenoids (Holland and Palacco, 1994). These rod-shaped facultative methylotrophs are widely distributed in nature but are mostly well known for being associated with plants (Araujo et al., 2002; Corpe, 1985; Holland and Palacco, 1994; Lidstrom and Christoserdova, 2002; Lodexyckx et al., 2002a). *Methylobacterium* strains have been shown to colonize the intercellular spaces and/or vascular tissues of plants as endophytes (Idris et al., 2004; Araujo et al., 2002; Lodexyckx et al., 2002b) and have also been found as endosymbionts within the cells of the buds of Scotch pine (Pirttila et al., 2000).

Bacteria of the genus *Methylobacterium* have been reported to be non-pathogenic to their plant hosts (Rughia et al., 2006). The host plants provide methanol or methylated pectin as nutrients for their host bacteria, while the bacteria can benefit the plants in different ways; such as stimulating seed germination and plant development, probably due to hormone or vitamin production (Basile et al., 1985; Hiraish et al., 1995). In addition, some strains showed 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity potential resulting in improved stress tolerance by plants (Idris et al., 2004). *Methylobacteria* can also contribute to a better iron nutrition of plants by producing the siderophores (Bar-Ness et al., 1992; Idris et al., 2004). In addition, evidence exists that *Methylobacteria* contribute to the flavour development of strawberries (Zabetakis, 1997).

Time course of bioflocculant production

The correlation between cell growth, pH and flocculating activity was investigated over a growth period of 10 days. The flocculating activity of the bioflocculant increased with cell growth and an optimum activity of more than

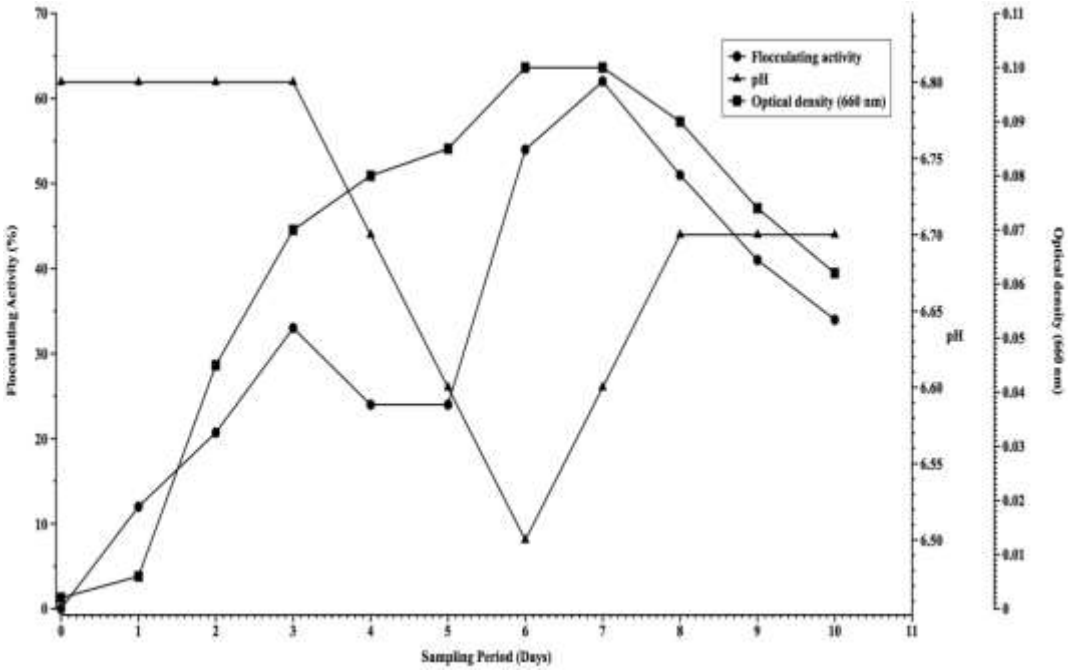

Figure 2. Time course assay of *Methylobacterium* sp. Obi showing correlation between cell growth (OD_{660nm}), pH and flocculating activity.

60% was obtained after 6 days of cultivation (Figure 2). Similar results were reported for *Bacillus* sp. by Feng and Xu (2008) and Xiong et al. (2010). A decrease in flocculating activity with a corresponding decrease in pH levels from 6.8 to 6.5 was observed in the first 6 days of growth (Figure 2). The decrease in pH is thought to be due to the production of organic acids either from glucose metabolism or the presence of organic acid components in the bioflocculant polymer being produced (Dermilim et al., 1999). An increase in flocculating activity was observed after a 5 day cultivation period and reached a maximum of 62% at an optimum pH of 6.6 at 7 days of cultivation (Figure 2). After seven days of cultivation, the pH stabilized at 6.7 with a corresponding sharp decrease in flocculating activity (Figure 2). The initial pH of the culture medium determines the electric charge of the cells and the oxidation-reduction potential which can affect nutrient absorption and enzymatic reaction (Nakata and Kurane, 1999; Salehizadeh and Shojaosadati, 2001). According to Tago and Aida (1977), this observed sharp decrease in activity may be attributed to the action of a bioflocculant-degrading enzyme being produced by the microorganism.

Several studies add been carried out on different bacterial strains, some of which corroborate the findings of this present investigation while some do not. Studies have carried out to investigate the effect of pH on flocculating activity of extracellular biopolymeric flocculants (EBFs). The flocculating activity of *Bacillus* sp. PY-90 is high in acidic pH (pH 3 -5) (Yokoi et al., 1995), whereas the maximum activity of the flocculant produced by *Enterobacter* sp. BY-29 is observed at pH 3 and the activity decreases with increasing pH (Yokoi et al., 1996). Shimofuruya et al. (1996) reported a flocculant produced by *Streptomyces griseus* that is active in acidic conditions ranging from pH 2 to 6 and with the maximum activity observed at pH 4. *Rhodococcus erythropolis* produced an EBF that is active at neutral pH (Kurane et al., 1994), whilst the flocculant produced by *Paceilomyces* sp. show maximum activity at the pH range of 4.0 - 7.5 (Takagi and Kadowaki, 1985). The flocculating activity of *Aspergillus sojae* increased when the pH exceeded 7 (Nakamura et al., 1976b) and the maximum flocculating activity of *Aspergillus* sp. JS-42 is reported in the pH range of 3 - 8 (Nam et al., 1996).

Effects of carbon and nitrogen sources on bioflocculant production

Carbon and nitrogen sources have been reported to have an effective role in enhancing the production of bioflocculant by microorganisms (Nakamura, 1976d). The composition of the production medium was supplemented with different carbon and nitrogen sources to optimize bioflocculant production. The effects of glucose, lactose, sucrose, fructose and starch were investigated. From results obtained, it was evident that glucose supported the highest bioflocculant production with an optimum flocculating activity of 72% at $p \leq 0.05$ compared to that of lactose (42%), sucrose (31%) and starch (11%). Fructose was the least preferred carbon source by

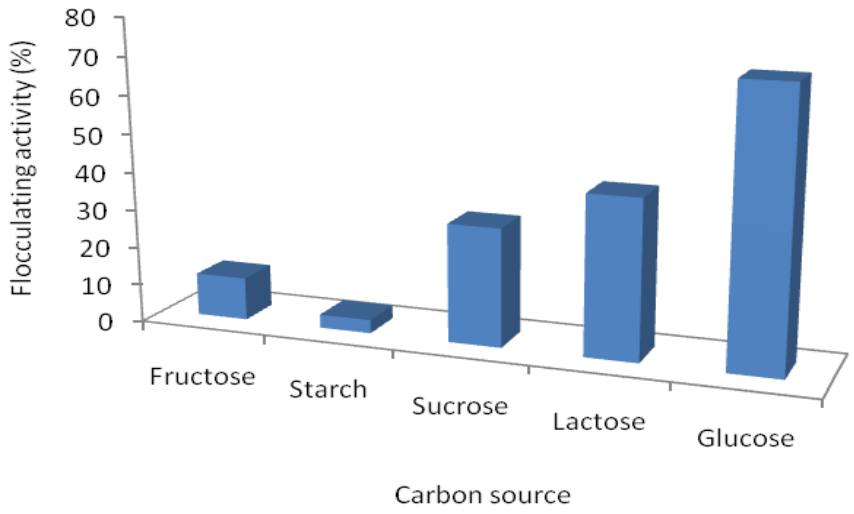

Figure 3. Effect of carbon source on bioflocculant production.

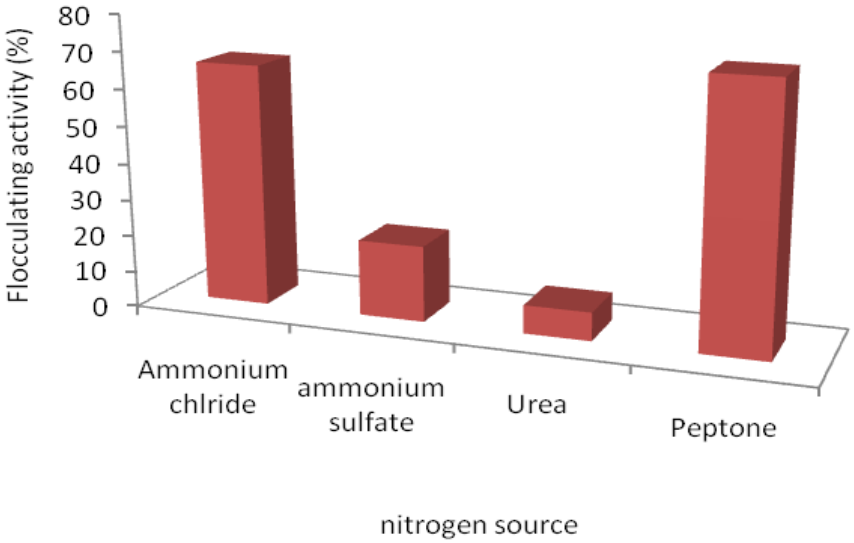

Figure 4. Effect of nitrogen source on bioflocculant production.

Methylobacterium sp. Obi and resulted in a low flocculating activity of less than 10% (Figure 3). Overall, the results reveal that glucose was the best carbon source for bioflocculant production by *Methylobacterim* sp. Obi, and the flocculating activity (in a kaolin suspension) was 72% after 7 days. Glucose has been reported as a preferred carbon source in previous studies on bioflocculant production by various microorganisms. Patil et al. (2009) reported that the bioflocculant produced by *Bacillus subtilis* is enhanced by glucose and sucrose as carbon sources. In the case of *Rhodococcus erythropolis*, glucose and fructose enhance elongation of the cells and the production of the bioflocculant (Kurane et al., 1991).

The production medium was further optimized for production of bioflocculant by supplementing it with different nitrogen sources. The effects of the following nitrogen sources on bioflocculant production were investigated: peptone, ammonium chloride, ammonium sulphate and urea. Although, they are all of natural nitrogen sources, only peptone and ammonium chloride showed significant support for bioflocculant production. Peptone resulted in a flocculating activity of 72% which was marginally better than 66 and 21% ($p \leq 0.05$) obtained with ammonium chloride and ammonium sulphate respectively (Figure 4). According Nakamura et al. (1976b) in the presence of certain inorganic nitrogen compounds (for example, ammonium chloride, ammonium nitrate, and ammonium sulfate), the mycelial growth is poor and

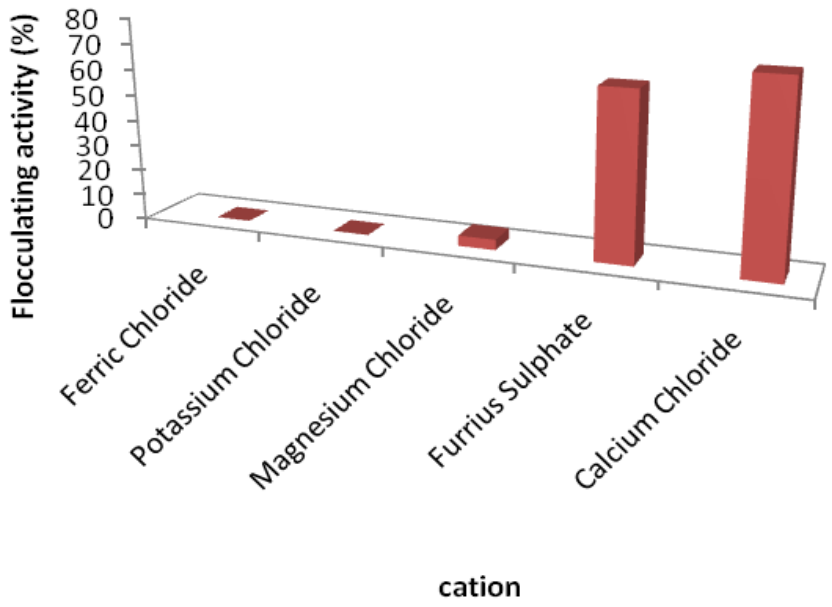

Figure 5. Effect of cations on flocculating activity.

and no flocculating activity could be detected.

Urea, with a flocculating activity of 8% was the least preferred nitrogen source (Figure 4). Therefore peptone remains the only suitable source of nitrogen for bioflocculant production by *Methylobacterium* sp. Obi. On the contrary, Nakumura et al. (1976b) reported that the production of the bioflocculant by *Aspergillus sojae* is enhanced when casein, polypeptone and glutamic acid were added to the medium.

Effect of cations on flocculating activity

Cations can neutralize negative charges of both polysaccharide and suspended particles and increase the adsorption of polysaccharide onto suspended particles (Wu and Ye, 2007). These cations stimulate flocculation by destabilizing the negative charge of the kaolin particle (Gong et al*.,* 2008). Divalent cations (Ca^{2+}, Fe^{2+}) were found to be more effective in stimulating flocculating activity than monovalent (K^{+}) and trivalent (Fe^{3+}) cations (Figure 5). Flocculating activities of 72 and 63% ($p \leq 0.05$) were obtained with Ca^{2+} and Fe^{2+}, respectively, whilst an insignificant activity of 4% was obtained with Mg^{2+}, Fe^{3+} and K^{+} showed no stimulation of flocculating activity (Figure 5).

As reported in previous studies, the Ca^{2+} ion enhances both the cell growth and the flocculating activity of *Paecilomyces* sp. (Takagi and Kadowaki, 1985). Good floc growth has been observed for *Kluyvera cryocrescens* in a medium containing a low concentration of Ca^{2+}, but flocs was not formed in the absence of Ca^{2+} (Kakii et al., 1990). These results reveal that Ca^{2+} and the cell surface are involved in bioflocculation (Endo et al., 1976). Salt ions, such as, Ca^{2+}, Co^{2+}, $Sr^{2+,}$ Mg^{2+}, Mn^{2+}, and Al^{3+} promote flocculation of *Kluyveromyces marxianus* cells, but not as efficiently as Fe^{2+} and Sn^{2+} (Sousa et al., 1992).

Effect of pH on flocculating activity

The pH is said to have a major influence on flocculating activity (Yokoi et al*.,* 1996). In the case of *Corynobacterium xerosis*, the flocculant is produced at relatively low pH (Esser and Kues, 1983). The mycelial growth of *Aspergillus sojae* is enhanced when the pH of the culture is controlled at 6 and no flocculating activity is observed in the cultures grown at pH 8 (Nakamura et al., 1976d). The flocculant production by *Rhodococcus erythropolis* is higher at alkaline pH values of 8.0 - 9.5 than at other pH values (Kurane and Matsuyama, 1994).

The effect of pH on flocculating activity of the bioflocculant produced by *Methylobacterium* sp. Obi was measured at pH values ranging from 3, 4, 5, 6, 7, 8, and 9, with the maximum activity peak of 72% obtained at an optimum pH of 7 after which a gradual decrease in activity was observed (Figure 6). Li et al. (2008) reported that at low pH, both bioflocculant and kaolin particles are likely to absorb hydrogen ions (H^{+}), which weakened the forming of complexes between bioflocculant molecules and kaolin particles mediated by Ca^{2+}. Similarly, hydroxide ions (OH^{-}) interfered with the combination of the flocculant molecules and kaolin particles at high pH values, resulting in lower flocculating activity. The mediating effect of Ca^{2+} appeared to be the strongest at

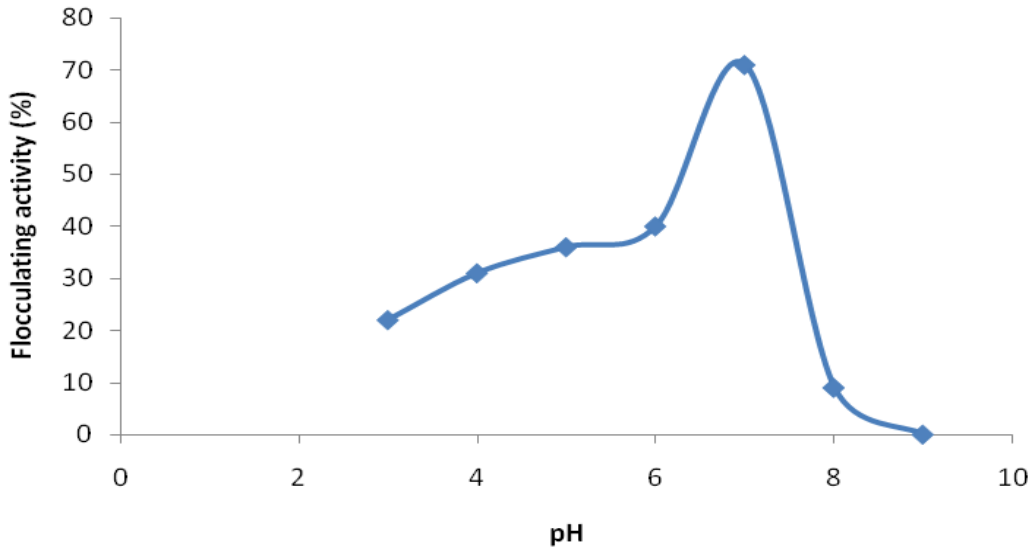

Figure 6. Effect of initial pH on bioflocculantion suspension by *Methylobacterium* sp. Obi.

neutral pH values (Li et al., 2008).

Composition analysis of the bioflocculant

Studies carried out have shown that the chemical nature of bioflocculants produced by different microorganisms differs (ABD-EL-Haleen et al., 2008). Several types of bioflocculants have been reported including polysaccharides, proteins, lipids, glycolipids, and glycoproteins (Zhang et al., 2002; Li et al., 2003). Phenol sulphuric acid determination of total carbohydrates indicated the presence of a polysaccharide as a major component of the bioflocculant produced by *Methylobacterium* sp. Obi with no protein peak detected by the Lowry-Folin assay. Similar results were obtained with *Serratia ficaria* (Gong et al., 2008), *Vagococcus* sp. W31 (Jie et al., 2006), *Klebsiella mobilis* (Wang et al., 2007). The total carbohydrate concentration of bioflocculant produced by *Methylobacterium* sp. Obi was measured at 23 mg/ml.

Conclusion

The strain *Methylobacterium* sp. Obi has proven to be an important producer of bioflocculant composed mainly of polysaccharide. Bioflocculant production by the bacterium was optimal at neutral pH and in the presence of glucose, peptone and Ca^{2+} as sole sources of carbon, nitrogen and cations respectively. A detailed characterization of the bioflocculant as well as establishment of process conditions for pilot scale production of the bioflocculant is necessary and is a subject of on-going investigation in our group.

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