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OPTIMISATION OF THE BIOFLOCCULANT PRODUCED BY *PANTOEA* SP., A NOVEL BACTERIUM ISOLATED FROM MARINE SEDIMENT

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Abstract

The search for safe and more eco-friendly flocculants for treatment of water and wastewater has led to the screening of microorganisms with potential to produce bioflocculants. An organism that secretes large amounts of bioflocculant was isolated from a sediment sample collected from the beach at Mtunzini on the coast of northern Kwazulu-Natal, South Africa. From detailed morphological and biochemical analysis, the isolate was identified as *Pantoea* sp. with 91% probability. Cultivation studies revealed that the isolate produced bioflocculant optimally with sucrose as a source of carbon (92.4% flocculating activity), inoculum size of 3% (v/v), at an initial pH of 6.0, with Ca²⁺ as an aid to coagulation, and a production time of 96 hrs. *Pantoea* sp has been noticed to have great potential as a producer of bioflocculant due to its ability to yield high flocculating activity at varied cultivation conditions. Thus, further studies on the characterisation and applications of the bioflocculant produced by this organism is recommended to underscore the biotechnological importance of this species as a producer of secondary metabolite that could be useful in water and wastewater treatment.

Keywords: *Pantoea* sp., Bioflocculant, Production, Flocculating activity, Cultivation

Introduction

Bioflocculation has been defined as a dynamic process resulting from the synthesis of extracellular polymers by living cells in order to clarify suspended particles in solution (He *et al.*, 2002; Gao *et al.*, 2006; Piyo *et al.*, 2011). The physico-chemistry of bioflocculation is determined by the genetic make-up of the organisms involved (Dugan, 1975). This has aroused great interest among researchers all over the world seeking to isolate and screen new microorganisms with bioflocculant-producing potential (Ugbenyen *et al.*, 2012).

Chemical flocculants have been used over the years for many downstream industrial processes requiring dewatering by clarification of solutions containing suspended particles and because they are inexpensive and readily available (Nakata and Kurane, 1999; Salehizadeh and Shojaosadati, 2001; Ugbenyen *et al.*, 2013). However, some chemical flocculants are hazardous by virtue of their carcinogenic effects – in the case of polyacrylamide for example (Dearfield and Abermathy, 1988) – and neurotoxic such as polyaluminium chloride (Kowall *et al.*, 1989). It has therefore become imperative to look for substitutes that are eco-friendly.

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Biofloculants are essentially biopolymers produced by microorganisms after their exponential growth phase. They have been reported to be safe (inert) and highly biodegradable (Li *et al.*, 2009; Liu *et al.*, 2010; Ugbenyen *et al.*, 2014).

The marine habitat is one of the most under-exploited environments as a source of substitutes for biofloculants; however, it is reported to be responsible for the production of half of all bioactive compounds discovered (Berdy, 2005).

The *Pantoea* genus comprises species that are associated with epiphytic plants. According to the literature, there are only seven species of this genus that have been distinguished; these are *Pantoea agglomerans*, *Pantoea terrea*, *Pantoea stewartii*, *Pantoea dispersa*, *Pantoea ananatis*, *Pantoea punctate* and *Pantoea citrea*, which have been isolated from various ecological niches such as plants, water, soil, animals and humans (Gavini *et al.*, 1989; Kageyama *et al.*, 1992; Mergaert *et al.*, 1993; Grimont *et al.*, 2005).

From our search of the literature this seems to be the first time this genus has been implicated in the production of biofloculant. The crude biofloculant produced by this organism is effective in clarifying kaolin clay in suspension, thereby showing great potential for applications in industrial biotechnology.

Materials and Methods

Description of study site

The beach at Mtunzini is a wide inlet along the northern KwaZulu-Natal coastal region of South Africa, located at 28°57'S and 31°45'E. It is bounded in the north by the Umlalazi River and in the south by the Amatikulu River. The beach plain is up to 436 m wide.

Sample collection and processing

Six different marine water and sediment samples were randomly collected from approximately 0–20 m directly offshore of the beach. The samples collected were transported in ice to the hydrology and microbiology laboratory of the University of

Zululand, KwaDlangezwa. Sediment samples were oven dried overnight at 30°C.

Isolation and screening of microorganisms

Biofloculant-producing microorganisms were isolated using an agar plate culture containing medium with the following composition: meat extract, 1.0 g; peptone, 5.0 g; yeast extract, 2.0 g; NaCl, 8.0 g; and agar, 15.0 g per litre of filtered sea water according to the method described by Jensen *et al.* (1991). These microorganisms were originally screened on the basis of colony morphology. Two loopfuls of bacterial colonies were then grown in 50 ml of screening medium described by Zhang *et al.* (2007) and Ugbenyen *et al.* (2012), with slight modifications, on a rotary shaker (160 rpm) at 30°C for 72 h. The medium contained (g/l of filtered natural sea water): glucose, 20; casein, 0.7; yeast extract, 0.5; KH₂PO₄, 2; K₂HPO₄, 5; NaCl, 0.1 and MgSO₄·7H₂O, 0.2. At the end of the incubation period, 2 ml of the fermentation broth was centrifuged (8 000 g, 30 min) to separate the cells, after which the cell-free culture supernatant was analysed for flocculating activity. Finally, one isolate with good flocculating activity for kaolin was selected for further study. The pre-culture was stored at 4°C and used for subsequent inoculations.

Flocculating activity experiment

Flocculating activity was measured according to the method described elsewhere (Kurane *et al.*, 1986; Zhang *et al.*, 2007; Cosa *et al.*, 2011; Ugbenyen *et al.*, 2012) with modifications. Briefly, 3 ml of 1% CaCl₂ and 2 ml of cell-free supernatant were added to 100 ml kaolin suspended solution (4 g/l) in a 250 mL flask. The mixture was stirred vigorously, poured into a 100 ml cylinder and allowed to stand for 5 min. The absorbance at 550 nm of the clarifying solution was measured spectrophotometrically. A control experiment was performed using the same method, but with fresh culture medium replacing the cell-free supernatant. The flocculating activity was calculated according to the equation:

$$\text{Flocculating activity (\%)} = [(B - A)/B] \times 100,$$

where *A* is the absorbance of the sample at 550 nm; *B* is the absorbance of the control experiment at 550 nm.

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Identification of microorganisms

Microorganisms were identified at the laboratory of Umgeni Water Pietermaritzburg (South Africa), on the basis of morphological and biochemical analysis using the VITEK 2 Systems Version: 06, 01.

Inoculum size experiment

An amount of 50 ml of production medium in 100 ml flasks was separately inoculated with 0.5, 1.0, 1.5, 2.0, 2.5 and 3 ml of the pre-culture, representing 1, 2, 3, 4, 5 and 6% inoculum size of the test bacteria, respectively, cultivated at 30°C and 160 rpm for 72 h. At the end of the incubation period, the fermentation broths were centrifuged (8 000 g, 30 min) to separate the cells. The cell-free culture supernatants were analysed for flocculating activity.

Effects of carbon and nitrogen sources

The effects of carbon sources on bioflocculant production by the *Pantoea* species were assessed. The carbon sources included glucose, sucrose, maltose, starch, galactose, fructose, sodium acetate and sodium carbonate. Nitrogen sources (namely, urea, peptone, casein and yeast extract) were assessed also for their effect on bioflocculant production. In the production medium, casein and yeast extract were

replaced with one of the nitrogen sources in equivalent amounts.

Effect of pH and cations

The effects of pH and cations on bioflocculant production were assessed in accordance with the description of Liu *et al.* (2010). The initial pH of the production medium was varied over a wide range of 3–12 using HCl and NaOH, whereas the cations that were varied included Li⁺, Na⁺, K⁺, Mg²⁺, Mn²⁺, Fe²⁺ and Al³⁺. With regard to the effects of cation assays, flocculant tests were conducted as described above, but with the CaCl₂ solution replaced by a solution of all the above cation chloride, and the flocculating activity was measured.

Time course assay

The composition of the medium for the time course assay was as described earlier, with slight modification (Zhang *et al.*, 2007; Ugbenyen *et al.*, 2012). The pre-culture was used to inoculate the production medium based on previously determined optimal growth conditions. Samples (2 ml) were withdrawn at 24-h intervals over a period of 120 h, centrifuged (8 000 g, 30 min) and the cell-free supernatant was used to determine the flocculating activity. The optical density (OD) of the broth at 660 nm was also recorded.

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) using the GraphPad PRISM 5 statistical package. A significance level of *p* < 0.05 was used. The experiments were carried out in duplicate.

Results

Identification of the microorganism.

Table 1: Biochemical analysis for identification of microorganism.

Test	Mnemonic	Result
Ala-Phe-Pro-arylamidase	APPA	(-)
Adonitol	ADO	(-)
L-Pyrrolydonyl-arylamidase	PyrA	(+)
L-Arabitol	IARL	(-)
D-Cellobiose	dCEL	(-)
Beta-galactosidase	BGAL	(+)
H ₂ S production	H ₂ S	(-)
Beta-N-acetyl-glucosaminidase	BNAG	(-)

	Glutamyl-arylamidase pNA	AGLTp	(-)
	D-Glucose	dGLU	(+)
	Gamma-glutamyl-transferase	GGT	(-)
	Fermentation glucose	OFF	(-)
	Beta-glucosidase	BGLU	(+)
Page	D-Maltose	dMAL	(+)
3069	D-Mannitol	dMAN	(+)
	D-Mannose	dMNE	(+)
	Beta-xylosidase	BXYL	(+)
	Beta-alanine arylamidase pNA	BAlap	(-)
	L-Proline arylamidase	ProA	(-)
	Lipase	LIP	(-)
	Palatinose	PLE	(-)
	Tyrosine arylamidase	TyrA	(-)
	Urease	URE	(+)
	D-Sorbitol	dSOR	(-)
	Saccharose/sucrose	SAC	(+)
	D-Tagatose	dTAG	(-)
	D-Trehalose	dTRE	(+)
	Citrate(sodium)	CIT	(-)
	Malonate	MNT	(-)
	5-Keto-D-gluconate	5KG	(-)
	L-Lactate alkalisation	ILATk	(-)
	Alpha-glucosidase	AGLU	(-)
	Succinate alkalisation	SUCT	(-)
	Beta-N-acetyl-galactosaminidase	NAGA	(-)
	Alpha-galactosidase	AGAL	(-)
	Phosphatase	PHOS	(-)
	Glycine arylamidase	GlyA	(-)
	Ornithine decarboxylase	ODC	(-)
	Lysine decarboxylase	LDC	(-)
	L-Histidine assimilation	IHISa	(-)

(+) Positive for test; (-) negative for test.

Inoculum size: The result of the inoculum size experiment on flocculating activity of the bioflocculant is shown in Figure 1.

Carbon sources: The effect of various carbon sources on bioflocculant production is represented in Figure 2.

Nitrogen sources: The utilisation of four different nitrogen sources (at 1.2 g/l) by the test bacteria for the production of bioflocculant is shown in Figure 3. **pH:** The effect of the

initial pH of the culture medium on bioflocculant production is presented in Figure 4. **Metal ions:** Different metal ions stimulated flocculating activity by *Pantoea* species above 65%, except for Li⁺, which completely inhibited bioflocculant production (Table 2). **Time course assay:** Figure 5 reveals the time course of bioflocculant production of *Pantoea* species.

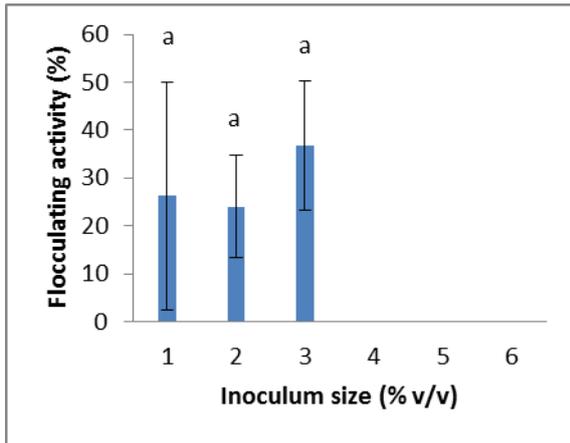


Figure 1: Effect of inoculum size on bioflocculant production by *Pantoea* species.

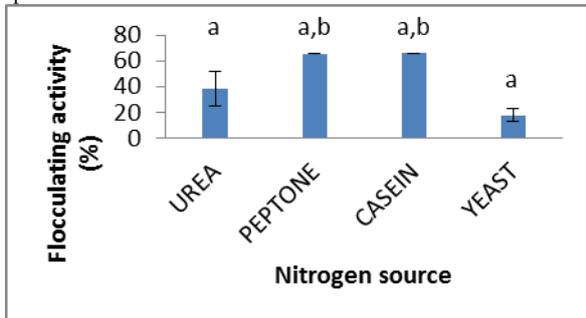


Figure 3: Effect of nitrogen sources on bioflocculant production by the bacteria. Activities indicated by bars with different letters were significantly different ($p < 0.05$) from each other.

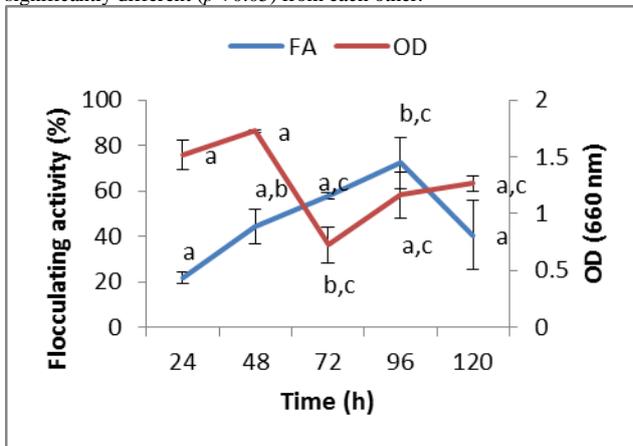


Figure 5: Time course of bioflocculant production by the bacteria. Activities corresponding to data points marked by different letters are significantly different ($p < 0.05$) from each other. FA, flocculating activity; OD, optical density.

Discussion

Isolation of microorganisms is important for characterising bacterial taxonomy and function in the search for new microbiological products (Alain and Querellou, 2009). In the study reported here a marine bacterium identified as *Pantoea* sp. was isolated with potential to produce bioflocculant. It was observed that the

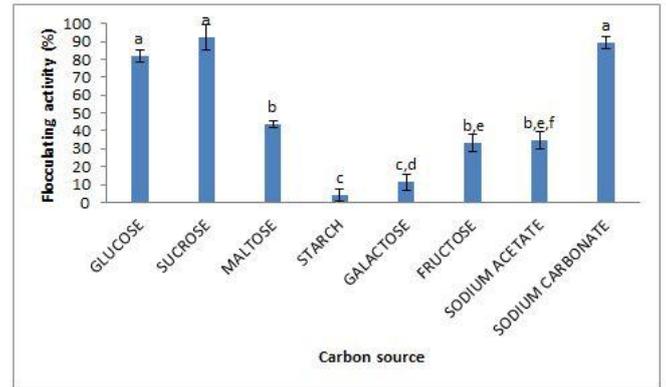


Figure 2: Effect of carbon sources on bioflocculant production by *Pantoea* bacteria. Activities indicated by bars with different letters were significantly different ($p < 0.05$) from each other.

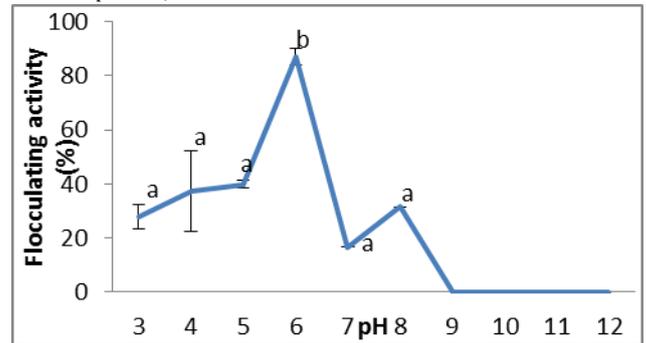


Figure 4: Effect of pH on bioflocculant production by *Pantoea* cultures. Activities corresponding to data points marked by different letters are significantly different ($p < 0.05$) from each other.

Table 2. Effect of metal ions on bioflocculant production of *Pantoea* sp.

Cation	Flocculating activity (%)
Li ⁺	0.0 ^a
Na ⁺	74.6 ± 8.5 ^{b,c}
K ⁺	71.5 ± 6.9 ^{c,d}
Mg ²⁺	68.8 ± 5.6 ^{b,c,d}
Ca ²⁺	82.0 ± 3.6 ^{b,c,d,e}
Mn ²⁺	83.8 ± 6.4 ^{b,c,d,e,f}
Fe ²⁺	84.5 ± 3.4 ^{b,c,d,e,f,g}
Al ³⁺	76.3 ± 3.4 ^{b,c,d,e,f,g,h}

Activities marked by different letters were significantly different ($p < 0.05$) from each other.

extracellular product from this organism's fermentation broth showed strong flocculating activity of kaolin suspension (82%). Due to the importance of inoculum size on cell growth of microorganisms (Zhong et al., 1995; Kallos and Behie, 1999), it was observed that an inoculum size of 3% (v/v) resulted in the highest flocculating activity. Further increases in

inoculum size showed zero flocculating activities (Figure 1). This conformed to the range reported for bioflocculant production by *Citrobacter* sp., *Proteus mirabilis* and *Bacillus* sp. Gilbert (Jang *et al.*, 2001; Xia *et al.*, 2008; Ugbenyen *et al.*, 2014).

Various other factors, such as the carbon and nitrogen sources for cultivation of the organisms, variation of initial pH of the culture medium and the effect of metal ions, are important for optimal bioflocculant production (Liu *et al.*, 2010; Li *et al.*, 2009; Piyo *et al.*, 2011; Ugbenyen *et al.*, 2013). These were assessed to establish optimum culture conditions for bioflocculant production by the *Pantoea* species.

Figure 2 shows that the *Pantoea* cultures effectively utilised glucose, sucrose and sodium carbonate as carbon sources for bioflocculant production, with flocculating activities of more than 80% – sucrose was the most successful at 92%. The corresponding results for maltose, fructose, sodium acetate, galactose and starch were less than 45%.

Related findings showed that *Corynebacterium glutamicum*, Mutator strain NIM-192, *Bacillus* sp. F19, and *Bacillus licheniformis* X14 all favoured the use of sucrose for bioflocculant production (He *et al.*, 2004; Li *et al.*, 2008; Zheng *et al.*, 2008; Li *et al.*, 2009).

It was observed that peptone and casein, when used respectively as the sole sources of nitrogen, were good producers of bioflocculant as revealed by their corresponding flocculating activities (65%) (Figure 3). Urea and yeast extract were poorer sources of nitrogen for the bacteria when used on their own (Figure 3). The corresponding flocculating activity for *Bacillus* sp. Gilbert, using peptone and casein as the sole sources of nitrogen, was about 70% (Ugbenyen *et al.*, 2014). Other investigators have reported good bioflocculant production with peptone and casein as nitrogen sources (Liu and Cheng, 2010, Deng *et al.*, 2005).

Several reports indicated that the initial pH of the culture medium affects bioflocculant production of microorganisms with this potential; this might be due to the claims that the electrical charge on cells, as well as the oxido-reduction potential that affects absorption of nutrient and other enzymatic reactions of cells, is dependent on the

initial pH of the culture medium (Nakata and Kurane, 1999; Xia *et al.*, 2008). The bacterial productivity was optimal at a pH of 6 (flocculating activity 87%). It was poor at pH 3–5, resulting in flocculating activities of less than 40%. Furthermore, a highly alkaline pH of 9–12 also did not favour bioflocculant production (Figure 4). This is in agreement with the work of Wang *et al.* (2007) who observed that bioflocculant production by *Klebsiella mobilis* was optimum (95%) at pH 6.

As regards the effects of metal ions on the flocculating activity of the bioflocculant, the divalent cations Ca^{2+} , Mn^{2+} and Fe^{2+} were found to be more effective than the monovalent or trivalent cations like K^+ , Na^+ and Al^{3+} (Table 2). The highest flocculating activity (85%) was observed with Fe^{2+} . This also confirmed the studies of Lee *et al.* (1995) who reported that the flocculating activity of a bioflocculant from *Arcuadendron* sp. TS-49 was highest with Fe^{2+} and Fe^{3+} as cations.

The OD_{660} graph, which represents cell growth during the cultivation period, shows a steady increase for the first 48 h then a sharp decrease at 72 hrs (Figure 5). However, the activity of the bioflocculant produced during this period increased and reached its maximum value of 72.2% after 96 hrs then declined sharply.

The growth profiles of many other bioflocculant-producing microorganisms have been reported. According to Shih *et al.* (2001), bioflocculant production by *Bacillus licheniformis* occurred in the growth phase and not during cell lysis. Lu *et al.* (2005) reported that the bacterium *Enterobacter aerogenes* produced bioflocculant and reached its maximum flocculating rate in the early stationary phase of cell growth. For *Agrobacterium* sp. M-503, a rapid increase in flocculating activity was observed in the log phase and remarkable flocculating activity was detected in the stationary phase of growth (Li *et al.*, 2010), these reports are in congruent with our study.

Conclusions

Isolate of *Pantoea* species from sediment samples from the beach at Mtunzini produced bioflocculants with promising potential. We report here the culture conditions under which

their production potential can be optimised. We propose that the bioflocculant produced by this bacterium would make a good substitute for the harmful chemical flocculants commonly used and could be of value in biotechnology for the treatment of water and wastewater.

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Conflict of Interest

The authors declare that there is no conflict of interest

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