

Isolation and Identification of Novel Bioflocculant-Producing Bacteria from Palm Oil Mill Effluent

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Bioflocculants produced by microorganisms from waste stream are regarded as being environmentally safe since they are nontoxic and biodegradable. In this study, the potential of palm oil mill effluent (POME) – a wastewater from the palm oil milling process – as a source of bioflocculant-producing bacteria was assessed. Several bacterial strains were isolated from POME and screened for bioflocculant production using kaolin suspension as indicator. Culture broth was used to determine the flocculating activity of the isolated strains, thereby assessing their potential to produce bioflocculant. Of the 23 screened isolates, 13 showed capability to produce bioflocculant. Four such isolates having the highest flocculation rate were identified as the potential bioflocculant-producing bacteria via 16S rDNA as *Bacillus marisflavi* NA8, *Xanthomonas oryzae* NA9, *Stenotrophomonas daejeonensis* NA12 and *Bacillus toyonensis* NA23. Interestingly, there was a high diversity of bioflocculant-producing bacteria in POME ecosystem suggesting that POME can be a highly suitable substrate in bioflocculant production.

Keywords: Bioflocculant, Bioflocculant-producing bacteria, Palm oil mill effluent, Isolation, Molecular identification, Phylogenetic tree.

Microbial flocculants (bioflocculants) are flocculating substances, which are secreted in the culture broth by many microorganisms like bacteria, fungi, actinomycetes, yeast and algae. Bioflocculants are biopolymers that promote flocculation by formation of bridges between them and other particles resulting in the aggregation and precipitation of suspended particles (Deng *et al.* 2003). Bioflocculants have drawn an increasing interest to date because they are biologically

active, biodegradable, non-secondary polluting and harmless to the environment (Jia and Yu 2012). Bioflocculants have been applied in various industrial processes, including water and wastewater treatment (Elkady *et al.* 2011; Xiong *et al.* 2010), heavy metals removal (Gomma 2012; Batta *et al.* 2013), decolorization (Buthelezi *et al.* 2012; Li *et al.* 2013), synthesis of nanoparticles (Raveendran *et al.* 2013; Salehizadeh *et al.* 2012; Sathyanarayanan *et al.* 2013), mining (Zhang *et al.* 2012; Liang *et al.* 2010) as well as cell removal and biomass recovery (Kim *et al.* 2011; Wan *et al.* 2013; Ghosh *et al.* 2009).

Bioflocculant production by microorganisms has been studied from the perspective of biotechnological potential. Thus, a variety of bioflocculant-producing microorganisms

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(BPM) have been isolated from a wide diversity of ecosystems including soil, activated sludge, wastewater, river, sea and even extreme environment. Microbial genera that have been described to date in producing bioflocculant include: *Aspergillus* (Rajab Aljuboori *et al.* 2013), *Bacillus* (Ugbenyen *et al.* 2013; Zulkeflee *et al.* 2012; Sekelwa *et al.* 2011; Sathiyarayanan *et al.* 2013; Elkady *et al.* 2011; Abdul Aziz *et al.* 2011), *Enterobacter* (Lu *et al.* 2005), *Klebsiella* (Yang *et al.* 2012; Wang *et al.* 2007; Zhao *et al.* 2013; Buthelezi *et al.* 2012), *Paenibacillus* (Li *et al.* 2013; Aguilera *et al.* 2008; Kim *et al.* 2011; Yang *et al.* 2009), *Pseudomonas* (Gomma 2012), *Rhodococcus* (Guo *et al.* 2013; Peng *et al.* 2014), *Serratia* (More *et al.* 2012; Liang and Song 2009) and many others.

Table 1 lists some of the recently isolated BPM. In general, activated sludge can be considered as a recognized and suitable mixed culture source for BPM. Bioflocculation usually occurs in activated sludge during aerobic process (Salehizadeh and Yan 2014), e.g. *Achromobacter* sp., *Exiguobacterium acetylicum*, *Galactomyces* sp., *Klebsiella terrigena*, *Rhodococcus erythropolis* and *Solibacillus silvestris* were isolated from activated sludge (Batta *et al.* 2013; Buthelezi *et al.* 2012; Wan *et al.* 2013; Guo *et al.* 2013). In several occasion, *Aspergillus flavus*, *Bacillus amyloliquefaciens* and *Bacillus subtilis* were isolated from soil samples (Rajab Aljuboori *et al.* 2013; Song *et al.* 2012; Sathiyarayanan *et al.* 2013). Besides, BPM such as *Brachybacterium* sp., *Cellulomonas* sp. and *Streptomyces* sp. were also screened from freshwater environment (Nwodo *et al.* 2012; 2013; Nwodo and Okoh 2013), *Arthrobacter* sp. from volcanic rocks (Mabinya *et al.* 2012), *Bacillus clausii* from brewery wastewater (Adebayo-Tayo and Adebami 2014) and *Pseudomonas aeruginosa* from oil refinery waste (Gomma 2012).

The palm oil industry is one of the leading industries in Malaysia with production of 20.7 million tonnes of crude palm oil (CPO) from an oil palm planted area of 5.39 million hectares in 2014 (MPOB, 2014). However, such production has resulted a large amount of palm oil mill effluent (POME), estimated at nearly three times the quantity of CPO. For each tonne of fresh fruit bunches (FFB) processed, an approximate of 0.67 tonne of POME is generated. Hence, in 2014, about

67.28 million tonnes of POME was produced. POME is a highly polluting wastewater due to its high chemical oxygen demand (COD) and biological oxygen demand (BOD) (Nurul Adela *et al.* 2014). POME containing a variety of microbial community as indicated by the high BOD poses huge potential as a source for BPM. Notably, there were still relatively few BPM isolated from POME, e.g. *Chryseomonas luteola* (Syafalni *et al.* 2012) and *Staphylococcus cohnii* (Wong *et al.* 2012). More studies are required to exploit POME as a source to develop new bioflocculant. Hence, the aim of this study was to isolate and screen the BPM from POME and identify them to provide a better insight on the diversity of the microbial community in POME ecosystem.

MATERIALS AND METHODS

Sampling and site description

Twelve samples from different effluent sources i.e. raw, aerobic and anaerobic ponds were collected from three different palm oil mills. A brief description of samples is given in Table 2. The selected mills were located at Banting and Dengkil in Selangor and Labu in Negeri Sembilan, Malaysia. All samples were properly labelled according to their abbreviation, location and subsequently stored at 4°C and analysed within 24 hour.

Isolation of pure cultures

The cell counting technique in agar plates was carried out to determine the population density of each sample. The nutrient agar (NA) was used to isolate the bacteria from the samples. About 0.1 mL of diluted POME sample was spread onto the surface of NA plates and incubated at 37°C for 24 h. The number of colonies growing on NA surfaces were calculated and their morphologies thoroughly observed. Finally, the morphologically distinguished visible colonies from spread plates were isolated using a sterile inoculation loop and streaked onto new NA plates. The plates were then incubated for another at 37°C for 24 h. Several cycles of replating onto new NA plates were done in order to produce a pure single bacterial colony on their NA surfaces before being incubated.

Screening of bioflocculant-producing bacteria

Bacterial growth

For pre-growth culture, 100 mL of nutrient broth (NB) was prepared and equally transferred

into 10 Falcon tubes (10 mL each). One loopful of pure single colony from the selected plates were inoculated into 10 mL of nutrient enriched tubes as a mean to promote the microbial growth. The tubes were then incubated at 37°C for 6 h. The growth medium for bioflocculant production was prepared according to Zhang *et al.* (2007) consisting of glucose (20 g), MgSO₄·7H₂O (0.2 g), (NH₄)₂SO₄ (0.2 g), K₂HPO₄ (5 g), urea (0.5 g), yeast extract (0.5 g) and KH₂PO₄ (2 g) in a litre of distilled water at pH 6.5. The medium was sterilized by autoclaving at 121°C for 15 min. All the 10 mL of pre-growth culture were inoculated into a 250 mL flask with screw cap containing 100 mL of the growth medium and incubated at 37°C in a shaker at 150 rpm for 3 days. The culture broth was used to determine the bacterial flocculating activities.

Determination of flocculating activity

Kaolin clay was used as the test material to determine the flocculating activity of the produced bioflocculant according to Kurane *et al.* (1994) with minor modifications. Five grams of kaolin clay was suspended in 1 L of distilled water to make a suspension concentration of 5 g/L. One hundred millilitres of kaolin suspension, 3 mL of 1% (w/v) CaCl₂ and 2 mL of culture supernatant were added into a 250-mL flask. The mixture was agitated vigorously for 60 s, then poured into a 100-mL measuring cylinder and allowed to settle for 5 min at room temperature. The optical density (OD) of the clarifying supernatant was measured at 550 nm with a UV spectrophotometer (Genesys, Thermo Scientific, USA) and the resulting flocculating activity was determined as follows: Flocculating rate (%) = [(A-B/A)] × 100%
A and B = OD of the control and sample measured at 550 nm, respectively.

Identification of bioflocculant-producing bacteria **Morphological characteristics**

For identification, all the potential bioflocculant-producing bacteria were stained according to Gram-staining procedures (Bergey's Manual of Systematic Bacteriology) (Holt 1994) and their staining activities observed under light microscope at 100 × magnification with emulsion oil. The morphologies of the isolates from the agar plates were also observed and recorded. For scanning electron microscope (SEM) analysis, samples were dehydrated employing critical point drying (CPD) i.e. Leica CPD prior to observation

using Hitachi SU1510 SEM under scattered electron mode with 15 kV.

Molecular identification

Bacterial isolates that displayed high flocculating activities were identified by 16S rRNA sequencing which included PCR amplification of conserved regions of genomic DNA (gDNA) sample, purification of the amplicons and bi-directional sequencing of the PCR products. Amplicons were amplified using 16S universal primers containing primers (10 µM), dNTPs (10 mM), PCR buffer (2X), MgCl₂ (25 mM), *Taq* polymerase (1U/µL) and double-distilled water. The DNA thermal cycler used for amplification was programmed as follows: initial denaturation at 94°C 4 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 sec, elongation at 72°C for 5 min; and final extension step consisting of 72°C for 10 min. PCR product was electrophoresed on 1% agarose gel and exposed to ultraviolet light prior to observe amplified 16S rRNA, subsequently undergo purification using gel purification (QIAGEN, USA). DNA sequence was then matched using Basic Local Alignment Search Tool (BLAST) programme against deposited sequences in GeneBank database to identify the respective strains. The 16S rRNA gene sequences which obtained were first aligned by using the MUSCLE programme or Multiple Sequence Comparison by Log - Expectation (<http://www.ebi.ac.uk/Tools/msa/muscle/>). An evolutionary distance matrix was generated as described by Jukes and Cantor (Jukes & Cantor 1969). Evolutionary trees for the data set were interfered by the neighbour-joining method of Saitou and Nei (1987) by using the neighbor-joining programme of MEGA6 software (Tamura *et al.*, 2013). The stability of relationships was assessed by performing bootstrap analyses on 5000 resampling.

RESULTS AND DISCUSSION

Isolation of bacteria from POME

A total of 36 morphologically distinct bacterial strains were obtained from 12 different POME sources at three palm oil mills (Table 3). Only six of them came from raw POME, while 18 and 12 from aerobic and anaerobic ponds, respectively. The densest population of bacterial communities i.e. 2.8 × 10⁸ and 2.7 × 10⁸ cfu/mL were

found from anaerobic and aerobic ponds of the same mill, respectively. The lowest density of 1.0×10^4 cfu/mL was found in the raw POME of two mills (Table 2). Of these, only 23 colonies were successfully purified. Most of them appeared in white, cream and yellow on NA plates. These colonies were coded as NA1 to NA23 and were further evaluated for their abilities to produce biofloculant.

Table 1. List of isolated biofloculant-producing microorganisms (BPM) (2012-2015)

BPM	Source	Reference
<i>Achromobacter</i> sp.	Activated sludge	Batta <i>et al.</i> (2013)
<i>Arthrobacter</i> sp.	Volcanic rocks	Mabinya <i>et al.</i> (2012)
<i>Aspergillus flavus</i>	Soil	Rajab Aljuboori <i>et al.</i> (2013; 2015)
<i>Bacillus amyloliquefaciens</i>	Soil	Song <i>et al.</i> (2012)
<i>Bacillus clausii</i>	Brewery wastewater	Adebayo-Tayo and Adebami (2014)
<i>Bacillus licheniformis</i>	Soybean paste	Zhao <i>et al.</i> (2013)
<i>Bacillus subtilis</i>	Soil	Sathiyarayanan <i>et al.</i> (2013)
<i>Bacillus velezensis</i>	Brackish water	Zaki <i>et al.</i> (2013)
<i>Brachybacterium</i> sp.	Freshwater	Nwodo <i>et al.</i> (2013)
<i>Cellulomonas</i> sp.	Freshwater	Nwodo and Okoh (2013)
<i>Chryseomonas luteola</i>	Palm oil mill effluent	Syafalni <i>et al.</i> (2012)
<i>Citrobacter</i> sp.	Kitchen drain	Kimura <i>et al.</i> (2013)
<i>Cobetia</i> sp.	Marine sediments	Ugbenyen <i>et al.</i> (2012)
<i>Exiguobacterium acetylicum</i>	Activated sludge	Buthelezi <i>et al.</i> (2012)
<i>Galactomyces</i> sp.	Activated sludge	Wan <i>et al.</i> (2013)
<i>Klebsiella terrigena</i>	Activated sludge	Buthelezi <i>et al.</i> (2012)
<i>Pseudomonas aeruginosa</i>	Oil refinery waste	Gomma (2012)
<i>Rhodococcus erythropolis</i>	Activated sludge	Guo <i>et al.</i> (2013)
<i>Solibacillus silvestris</i>	Activated sludge	Wan <i>et al.</i> (2013)
<i>Staphylococcus cohnii</i>	Palm oil mill effluent	Wong <i>et al.</i> (2012)
<i>Streptomyces</i> sp.	Freshwater	Nwodo <i>et al.</i> (2012)

Table 2. Description of palm oil mill effluent samples

Sample code	Sample site	Sample source	HRT (days)	pH	Temperature (°C)	Colony forming unit (cfu/mL)
JR	Jugra	Raw effluent	-	4.63	80-90	1.0×10^4
JA2	(Banting)	Aerobic pond 2	7.5	8.47	38.5	1.9×10^7 $- 2.1 \times 10^7$
JAN4		Anaerobic pond 4	15	7.78	39.0	1.0×10^7
SR	Sri Ulu Langat	Raw effluent	-	4.64	80-90	1.0×10^4
SA1	(Dengkil)	Aerobic pond 1	11	7.66	40.5	1.1×10^7 $- 3.2 \times 10^7$
SAN3		Anaerobic pond 3	5.75	5.20	44.5	7.7×10^7
SAN5		Anaerobic pond 5	5.75	5.87	44.5	2.0×10^7 $- 2.5 \times 10^7$
LR1	POMTEC	Raw effluent	-	4.73	80-90	5.2×10^5
LR2	(Labu)	Raw sludge pit	-	4.76	80-90	4.0×10^5
LA1		Aerobic pond 1	24	7.86	35.4	6.3×10^7 $- 2.7 \times 10^8$
LA2		Aerobic pond 2	24	7.86	35.4	3.1×10^5
LAN		Anaerobic pond 2	12	7.38	41.0	5.6×10^7 $- 2.8 \times 10^8$

Screening of bioflocculant-producing bacteria

Not all the 23 pure strains (NA1–NA23) were capable of producing bioflocculant. Interestingly, some of them had abilities to settle the kaolin particles to the bottom of the flasks compared to the control (Figure 1a and 1b) and some showed capabilities to flocculate the particles (Figure 1c and 1d). Initially, the kaolin particles were dispersed prior to flocculation. During the process of coagulation-flocculation, the scattered kaolin particles were probably knitted and

adsorbed onto the binding sites of the bioflocculants and thus aggregated, forming larger flocs and leading to rapid sedimentation due to gravity (Xiong *et al.* 2010; Sekelwa *et al.* 2013). After screening, only 13 of them (NA2, NA4, NA6, NA7, NA8, NA9, NA12, NA14, NA19, NA20, NA21, NA22 and NA23) showed considerable flocculating rate (>40%) which were regarded as the bioflocculant-producing bacteria (Figure 2). Finally, four promising isolates (NA8, NA9, NA12 and NA23) showing consistent flocculating rates

Table 3. Morphological characteristics of distinct bacterial strains from palm oil mill effluent

No	Source	Isolate code	Colony morphology					
			Size	Shape	Colour	Margin	Elevation	Surface
1	JR	NA1	Large	Irregular	Cream	Undulate	Flat	Glistening
2	JA2	NA2	Large	Irregular	White transparent	Undulate	Raised	Glistening
3	JA2	NA3	Small	Irregular	Yellow orange	Undulate	Flat	Glistening
4	JA2	NA4	Large	Irregular	White	Undulate	Flat	Dull
5	JA2	NA5	Small	Circular	Yellow	Entire	Raised	Glistening
6	JA2	NA6	Small	Irregular	Cream	Undulate	Flat	Glistening
7	JA2	-	Small	Circular	Orange	Entire	Flat	Glistening
8	JA2	NA7	Small	Irregular	Cream	Undulate	Flat	Dull
9	JA2	NA8	Large	Irregular	Yellow	Undulate	Flat	Glistening
10	JAN4	NA9	Large	Rhizoid	White transparent	Lobate	Raised	Glistening
11	SR	NA10	Small	Circular	White transparent	Entire	Raised	Glistening
12	SA1	NA11	Small	Circular	Cream	Entire	Flat	Glistening
13	SA1	NA12	Small	Circular	White	Entire	Flat	Glistening
14	SA1	-	Small	Circular	White transparent	Entire	Flat	Glistening
15	SA1	-	Small	Circular	Yellow transparent	Entire	Flat	Glistening
16	SA1	-	Small	Circular	White	Entire	Flat	Dull
17	SA1	-	Small	Circular	Cream	Entire	Flat	Glistening
18	SAN3	NA13	Small	Circular	Cream	Entire	Raised	Glistening
19	SAN3	-	Punctiform	Circular	White transparent	Entire	Raised	Glistening
20	SAN3	NA14	Small	Circular	White transparent	Entire	Flat	Glistening
21	SAN3	-	Small	Circular	White ring	Entire	Flat	Glistening
22	SAN3	NA15	Punctiform	Circular	White transparent	Entire	Flat	Glistening
23	SAN5	NA16	Small	Circular	White	Entire	Flat	Glistening
24	SAN5	NA17	Large	Irregular	White	Undulate	Flat	Dull
25	SAN5	-	Punctiform	Circular	White transparent	Entire	Raised	Glistening
26	SAN5	-	Punctiform	Circular	White transparent	Entire	Flat	Glistening
27	LR1	-	Small	Circular	White transparent	Entire	Flat	Dull
28	LR1	NA18	Punctiform	Circular	White	Entire	Flat	Dull
29	LR2	NA19	Small	Irregular	White	Undulate	Flat	Rough
30	LR2	NA20	Small	Irregular	Cream	Undulate	Flat	Rough
31	LA1	NA21	Large	Irregular	Yellow	Undulate	Flat	Rough
32	LA1	-	Small	Circular	White transparent	Entire	Flat	Mucoid
33	LA1	NA22	Small	Circular	White	Undulate	Flat	Rough
34	LA2	NA23	Large	Irregular	White	Undulate	Flat	Dull
35	LAN	-	Small	Circular	Yellow white	Entire	Flat	Mucoid
36	LAN	-	Small	Circular	White	Entire	Flat	Mucoid

(>60%) i.e. 65.8 ± 2.57 , 63.9 ± 0.64 , 76.2 ± 2.31 and 64.2 ± 2.57 , respectively were selected for identification.

All the four selected isolates gave higher flocculating rate (>60%) in the kaolin clay suspension at 48 h than 72 h of incubation (Table 4). This indicated that these bacteria had adapted comfortably and their growth multiplied to the fullest in the medium used before degenerated gradually thereafter. This was probably due to an increased enzymatic activity and cell lysis – a phenomenon commonly occurred within the bacteria cells upon reaching the beginning of the

late stationary phase (adaptive phase) i.e. at 48 h in this case (Rajab Aljuboori *et al.* 2013; Xiong *et al.* 2010). This was evidently demonstrated by NA12 with the highest flocculating rate (76.2%) followed by NA23 (66%), NA8 (63.4%) and NA9 (62.7%) at the early stationary phase. The increasing of cultivation time thereafter (72 h) showed, decreasing flocculating rate, indicated that these strains had reached their initial death phase.

Morphological characteristics by Gram-staining and SEM

From Table 3, the morphological characteristics of the four bacterial colonies were quite indistinguishable from the shape, surface appearance and elevation. However, the Gram-staining identification of the four potential bioflocculant-producing isolates showed purple and pink colour stains indicative of two Gram positive (NA8 and NA23) and the other two Gram-negative (NA9 and NA12) bacteria, respectively (Figure 3). The SEM showed that the Gram-positive bacteria (NA8 and NA23) appeared in rod shape (bacillus) in colony size of $0.5 \mu\text{m} \times 0.75\text{-}1.5 \mu\text{m}$ and $0.7 \mu\text{m} \times 1.0\text{-}1.5 \mu\text{m}$ (width x length), respectively while the Gram negative bacteria (NA9 and NA12) in round shape (cocci), with dimension of $0.75\text{-}1.0 \mu\text{m}$ and $0.5\text{-}0.75 \mu\text{m}$, respectively (Figure 4).

Sequence analysis and phylogenetic tree

The taxonomy of the genus is traditionally based on morphological

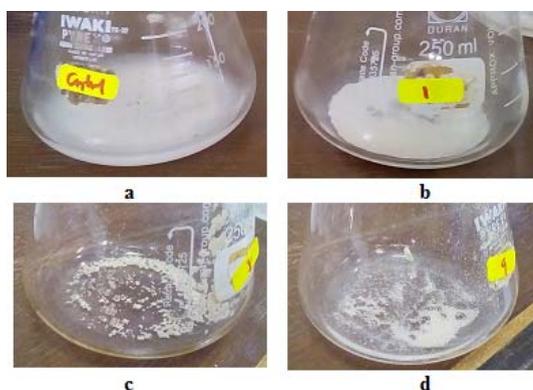


Fig. 1. Kaolin particles flocculated by isolated strains: (a) Control; (b) settled particles; (c) and (d) flocculated particles

Table 4. Characteristics and flocculating rate of the four bioflocculant-producing bacteria after re-screening at specific incubation periods

Isolate	Cell shape	Size (μm)	Gram-stain	Flocculating rate (%)	
				48 h	72 h
NA8	<i>Bacillus</i>	$0.5 \times 0.75 - 1.5^*$	+	63.4	55.7
NA9	<i>Cocci</i>	$0.75 - 1.0$	-	62.7	50.2
NA12	<i>Cocci</i>	$0.5 - 0.75$	-	76.2	75.3
NA23	<i>Bacillus</i>	$0.7 \times 1.0 - 1.5^*$	+	66.0	58.45

*width \times length

Table 5. Closest known bacteria to bioflocculant-producing bacteria isolates based on 16S rDNA sequences

Isolate	Nearest relative	Accession	bp length, status	Identity (%)
NA8	<i>Bacillus marisflavi</i>	NR118437	604, partial sequence	99
NA9	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	NR074938	602, complete sequence	99
NA12	<i>Stepnotrophomonas daejonensis</i>	NR115687	602, partial sequence	98
NA23	<i>Bacillus toyonensis</i>	NR121761	602, complete sequence	99

characteristics. However, difficulties were found among close groups as populations of the same genus. For specific identification, 16S rRNA of ribosomal gene was amplified using universal 16S primer. A single band with expected size ranging of 602–604 base pairs (bp) was observed from all isolates (Table 5). The GC contents of the 16S region of respective isolates were calculated as 53.5% (NA23), 55% (NA12), 53.8% (NA8) and 54.8% (NA9), respectively. It is envisaged that the bacterial cells with higher GC-content undergo autolysis, thereby reducing the longevity of the cell *per se* and it is believed that the GC content played a vital part in bacterial adaptation to temperature (Levin & Van Sickle 1976).

The phylogenetic trees constructed from 16S region sequences depicted a similar pattern between species and varies among genus (Figure 5). Interestingly, the phylogenetic tree constructed in this study, subdivided isolated bacterial into two distinct groups which showed a high level of genetic diversity. Interestingly, based on phylogenetic tree, isolates NA8 and NA23 were much closed related and clustered into same group. Isolates NA8 and NA23 was clearly shown to be the Gram-positive bacteria group together with *Bacillus* spp. Sequence analysis using Blastn using GeneBank database (<http://www.ncbi.nlm.nih.gov/>) predicted isolates NA8 and NA23 as *Bacillus toyonensis* (Accession

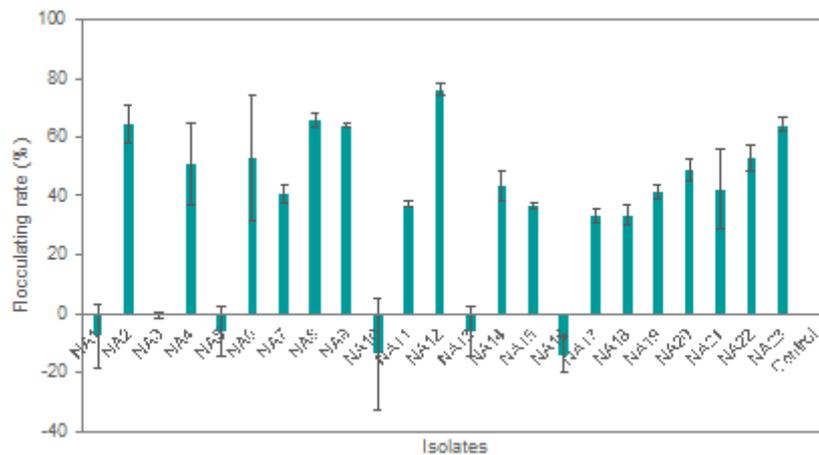


Fig. 2. Flocculating rate among isolated strains. Error bars indicate standard deviation of triplicate experiments

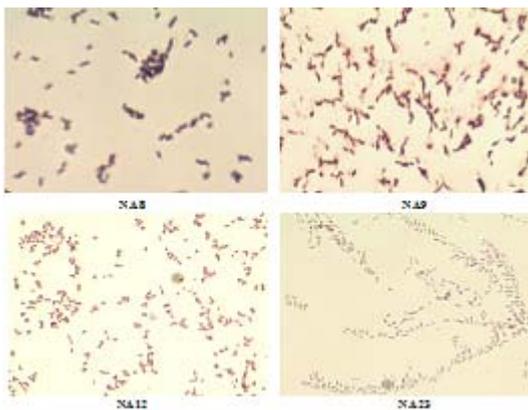


Fig. 3. Microscopic view of the four bioflocculant-producing bacteria identified by Gram-staining (at 100 × magnification)

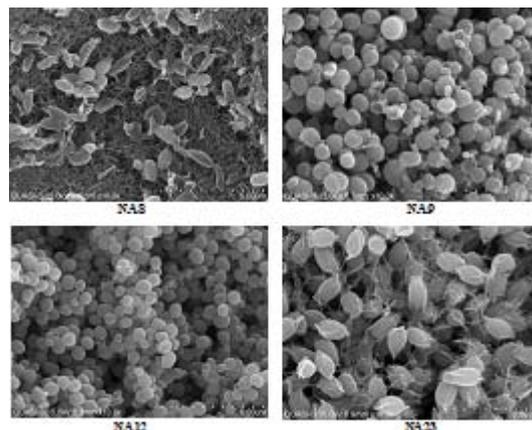


Fig. 4. The scanning electron microscope (SEM) images of the four bioflocculant-producing bacteria (at 10000 × magnification)

number NR121761) and *Bacillus marisflavi* (Accession number NR118437) both with identity of 99%, respectively.

Many researchers have managed to isolate bacteria belonging to the Bacillaceae family or more specifically *Bacillus* genus capable of secreting bioflocculant (Abdel-Aziz *et al.* 2011; Adebayo-Tayo and Adebami 2014; Deng *et al.* 2003; Elkady *et al.* 2011). In this study, two novel bioflocculant-producing *Bacillus* species were identified i.e. *B. marisflavi* and *B. toyonensis*. *Bacillus* can be obligate aerobes or facultative anaerobes. Both of the newly isolated *Bacillus* spp. in this study are obligate aerobic bacteria since they were isolated from aerobic ponds. Salehizadeh and Yan (2014) also reported that bioflocculation usually occurs during aerobic process.

Meanwhile, isolates NA12 and NA9 were clustered into the Gram-negative bacteria group closely divergence with *Pseudomonas jessenii* (outgroup). Both isolates NA12 and NA9 were found identical to *Stenotrophomonas daejeonensis* (Accession number NR115687) and *Xanthomonas oryzae* pv. *oryzae* (Accession number NR074938) both with similarity above 98%, respectively. These two genera i.e. *Xanthomonas* and *Stenotrophomonas* are also novel and not yet reported as bioflocculant producers. Both of them - *Xanthomonas oryzae* pv. *oryzae* and *Stenotrophomonas daejeonensis* - have been recognized as common plant pathogen (Hopkins *et al.* 1992).

Pairwise sequence alignment showed that both isolates shared 97.5% of homology by using

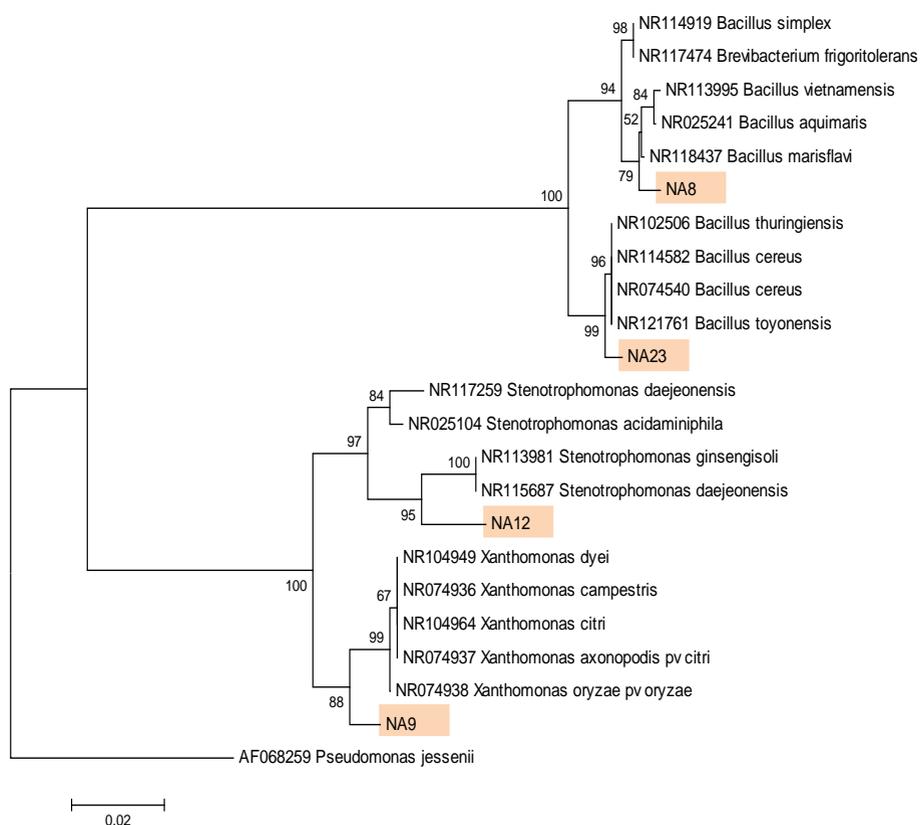


Fig. 5. Phylogenetic trees calculated from neighbor-joining depicting relationships among the most hits Blast against NCBI 16S ribosomal RNA sequences (Bacteria only) Database, excluding uncultured Bacteria bacterium (taxid: 77133). Branch lengths are proportional to nucleotide differences, and the numbers given at nodes are the percentage of frequencies with which a given branch appeared in 5000 bootstrap replications. Frequencies of bootstrap provided for branch receiving 50% or more support. The tree was rooted with *Pseudomonas jessenii* (Accession Number: AF068259) as an outgroup. The scale bar represents the sequence divergence.

LALIGN (<http://www.ebi.ac.uk/Tools/psa/lalign/nucleotide.html>). In addition, LALIGN analysis showed that these two isolates found to be similar at 95.3%, providing the information that they

shared highly conserved fragment yet identified in different genus. Conserved fragments of 16S sequences of all four isolates indicated by the shading of the consensus. Black shading box in

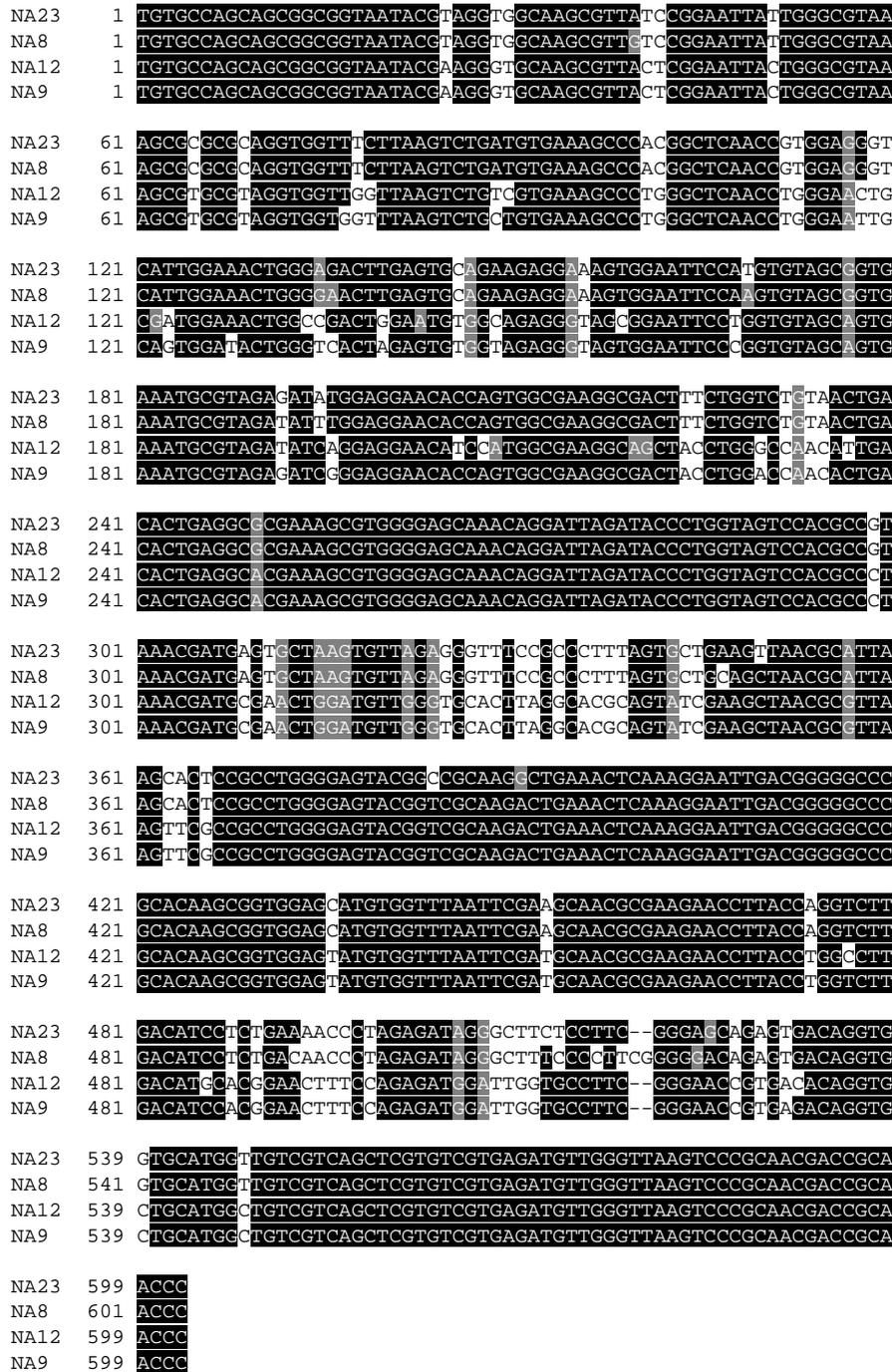


Fig. 6. Multiple sequence alignment of four isolates NA23, NA8, NA12 and NA9 showed conserved fragments using 16S sequence. Alignment was done using MAFFT and BoxShade programme.

the alignment indicated identical conserved regions, grey shading box indicated similar nucleotide fragments and completely unconserved showed in white shading (Figure 6). Multiple sequence alignment (MSA) was done using the MAFFT or Multiple Alignment using Fast Fourier Transform (<http://www.ebi.ac.uk/Tools/msa/mafft/>) against for all four isolates and pre-aligned sequences was then submitted in the BoxShade to present the alignment in shading form (http://embnet.vital-it.ch/software/BOX_form.html).

CONCLUSION

There was a high level of genetic diversity of bioflocculant-producing bacteria found in POME ecosystem. From this study, four novel bioflocculant-producing bacteria were successfully isolated and designated as *Bacillus marisflavi* NA8, *Xanthomonas oryzae* NA9, *Stenotrophomonas daejeonensis* NA12 and *Bacillus toyonensis* NA23. However, more studies are deemed necessary to (1) optimise the strains' culture conditions to enhance their flocculating performance and (2) reduce the production cost via fermenting them in wastewater. Continued discovery of new bioflocculants featuring yield increment and cost reduction will eventually outperform and able to compete with synthetic flocculants in bioremediation, leading to improved quality of the environment in the future.

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