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Screening and Production of Polyhydroxybutyrate (PHB) by Bacterial Strains Isolated from Rhizosphere Soil of Groundnut plants

(Penyaringan dan Pengeluaran Polihidroksibutirat (PHB) oleh Pencilan Strain Bakteria daripada Tanah Rizosfera Tumbuhan Kacang Tanah)

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ABSTRACT

Polyhydroxybutyrate (PHB) otherwise known as bioplastics are biodegradable materials that are accumulated in various microorganisms to serve as carbon and energy reservoirs and regarded as an attractive alternative to petroleum-derived plastics. Although research has been conducted on isolation of PHB-producing microorganisms from different ecological environments, few studies have been carried out on isolation of potential PHB-producing microorganisms from rhizosphere environment of groundnut plants, Arachis hypogaea *which can be regarded as a good environment for the isolation of potential PHB-producing microorganisms. In the present study, a total of twenty-one (21) bacterial strains were primarily screened and isolated from rhizosphere soil of a groundnut plant. Four bacterial isolates with maximum PHB-producing potential upon screening using submerged fermentation were selected for further studies. The fermentation pattern of PHB production was studied using different nutrient sources. The influence of agitation on PHB production was also studied. Mannitol stimulated maximum (6.076^a mg/mL) PHB production by* Bacillus *sp. 1; KNO_3 used as a limiting nutrient induced best (5.728^a mg/mL) PHB production by* Citrobacter *sp. and $MgSO_4.7H_2O$ supported maximum (5.972^a mg/mL) PHB production in* Enterococcus *sp. A low agitation speed of 150 rpm was found to support best (5.802^a mg/mL) PHB production by* Bacillus *sp.1. Findings from this study indicated that the isolated bacterial strains have high PHB- producing potential. The need to explore other environment harbouring microbial strains with high PHB-producing potential is paramount to the discovery of bioplastics with improved properties for potential industrial applications.*

Keywords: Arachis hypogaea*; bioplastics; polyhydroxybutyrate; Sudan-Black staining*

ABSTRAK

Polihidroksibutirat (PHB) atau dikenali sebagai bioplastik adalah bahan terbiodegradasi yang terkumpul di dalam pelbagai mikroorganisma untuk menjadi takungan karbon dan tenaga serta dianggap sebagai alternatif yang menarik kepada plastik daripada petroleum. Walaupun penyelidikan telah dijalankan ke atas pengasingan mikroorganisma penghasilan PHB dari persekitaran ekologi yang berbeza, beberapa kajian yang telah dijalankan ke atas pengasingan mikroorganisma penghasilan PHB yang berpotensi daripada persekitaran rizosfera tumbuhan kacang tanah, Arachis hypogaea *boleh dianggap sebagai persekitaran yang baik untuk pengasingan mikroorganisma yang berpotensi menghasilkan PHB. Dalam kajian ini, sejumlah dua puluh satu (21) strain bakteria telah ditapis dan diasingkan dari tanah rizosfera dan tumbuhan kacang tanah. Empat pencilan bakteria dengan potensi maksimum untuk penghasilan PHB melalui penyaringan fermentasi tenggelam telah dipilih untuk kajian lanjut. Corak fermentasi pengeluaran PHB dikaji menggunakan sumber nutrien berbeza. Pengaruh penggoncangan ke atas pengeluaran PHB juga dikaji. Manitol merangsang pengeluaran maksimum PHB (6.076^a mg/mL) dengan penggunaan* Bacillus *sp.1; KNO_3 sebagai penghad nutrien teraruh terbaik (5.728^a mg/mL) pengeluaran PHB oleh* Citrobacter *sp. dan $MgSO_4.7H_2O$ menyokong pengeluaran maksimum PHB (5.972^a mg/mL) dalam* Enterococcus *sp.. Kelajuan penggoncangan yang rendah (150 rpm) dilihat menyokong pengeluaran terbaik PHB (5.802^a mg/mL) oleh* Bacillus *sp.1. Hasil kajian ini menunjukkan bahawa strain bakteria yang dipencil mempunyai potensi tinggi dalam penghasilan PHB. Keperluan untuk meneroka persekitaran lain yang melindungi strain mikrob berpotensi tinggi dalam penghasilan PHB amat penting dalam penemuan bioplastik dengan sifat yang lebih baik untuk aplikasi industri yang berpotensi.*

Kata kunci: Arachis hypogaea*; bioplastic; pewarnaan Sudan-Hitam; polihidroksibutirat*

INTRODUCTION

The accumulation of synthetic, petroleum-derived plastics in the environment over the past decades has caused serious environmental problems because of their non-biodegradable nature. This has prompted the need to look for alternative plastics that are biologically degradable under appropriate conditions and environmentally friendly. Biodegradable plastics that are produced by living organisms such as plants, fungi and bacteria may provide a solution to these environmental problems (Pei

et al. 2011). Poly-3-hydroxybutyrate (PHB) is a polymer of 3-hydroxyybutyrate consisting of intracellular granules produced by prokaryotic microorganisms as energy and carbon storage during starvation. PHB has many applications such as for industrial use in packaging and cosmetic containers; and for agricultural, medical and pharmacological use as replacement for petrochemical polymers.

PHB is naturally synthesized by several bacterial strains and is accumulated as energy and carbon storage compounds, usually in excess of carbon source and limited nutritional factors such as nitrogen, phosphorous, potassium, oxygen, sulphur or magnesium. Several bacterial species such as *Ralstonia eutropha*, *Azotobacter* sp., *Pseudomonas* sp. and *Bacillus* sp. have been reported as potent producers of PHB and can be isolated from diverse environments namely; oil-contaminated soils, activated sludge, rhizosphere and hypersaline environments (Shodhganga 2011).

Generally, several factors such as culture temperature, pH and rate of agitation alongside both the excess of carbon and limitation of nitrogen need to be considered in the selection of microbial strains for PHB production (Saharan et al. 2014). PHB can be determined using a number of methods such as Sudan-black and Nile blue staining. Other methods for determination and quantification of PHB content include extraction using chloroform, sodium hydroxide, sodium hypochlorite or combinations of these solvents (Shodhganga 2011).

Much research has involved the isolation of promising PHB-producing microorganisms from different environments such as microbial mats, municipal sewage sludge and marine environments. However, few studies have been conducted on rhizosphere environment of leguminous plants. The root nodules of leguminous plants e.g. groundnut plant can be regarded as a good ecological environment for the isolation of potential PHB-producing microorganisms because plants interact with abundant and diverse group of bacteria present in the soil (Kumbhakar et al. 2012). Thus in this study, isolation and screening of bacterial strains with PHB-producing potential from rhizosphere of groundnut plant was conducted and the effect of some nutritional and physiochemical parameters on the growth and PHB production by the selected strains investigated.

Materials and Methods

Sample collection

Soil samples were collected from rhizosphere of groundnut plant in a farm garden in Tsaragi (9° 10' latitude and 4.5° 05'N longitude), Edu local government area of Kwara State, Nigeria. The samples were taken from 0-10 cm beneath the surface of different field area close to the root of the plants, collected inside sterile sample bottles and taken to the laboratory for analysis.

Isolation and generic level identification of bacterial strains

Serial dilution of the collected soil samples was conducted according to the method of Olutiola et al. (2000). A total of 1 mL of the diluents was pour plated on nutrient agar (NA) and incubated at 37°C for 24-48 h. Morphological appearances of the bacterial colonies on the Petri plates were observed and distinct colonies sub-cultured to obtain pure isolates which were then maintained on nutrient agar slants and stored at 4°C for further studies. The pure isolates were further identified by morpho-physiological and biochemical characterization (catalase, citrate, motility and carbohydrates fermentation) using Bergey's Manual of Determinative Bacteriology (Holt et al. 2000).

Screening of PHB-producing bacterial isolates

The isolates were screened primarily for PHB-producing potential by staining the isolates with Sudan-black stain and observed under a microscope using ×100 objectives. Isolates with PHB-producing potential were stained light to deep red in colour. The isolates were further screened for PHB production using submerged fermentation condition. The basal medium used for screening by submerged fermentation contained the following composition; glucose: 1% w/v, peptone: 0.5% w/v, NaCl: 0.25% w/v (Aarthi & Ramana 2010). Four isolates with high PHB-producing potential were selected for further work.

PHB production and extraction

PHB production was carried out by introducing 1 mL of 24 h old broth culture into 25 mL Mc Cartney bottles containing 20 mL basal medium with the same composition as the one used for screening by submerged fermentation (Aarthi & Ramana 2010). The inoculated basal medium was incubated on a rotary shaker at 120 rpm and at a temperature of 37°C for 24-48 h.

PHB extraction was done following the method of Aslim et al. (2002) with slight modification. The 48 h old growth medium of the isolates was centrifuged at 3000 rpm for 15 min after which the pellet was suspended in a known volume of sodium hypochlorite (NaOCl) and incubated at 37°C for 1 h to break the cell wall. The pellet suspension in NaOCl was centrifuged again at 3000 rpm for 15 min and the collected pellet washed by adding 5 mL of acetone: ethanol (1:1 v/v) to extract cell lipids and other molecules with the exception of PHB granules. PHB was extracted by adding 10 mL boiling chloroform in a water bath and then filtered. Excess chloroform was evaporated to obtain PHB crystals after which 10 mL of 98% H_2SO_4 was added and heated at 100°C for 10 min in a water bath. PHB was determined quantitatively as crotonic acid by measuring the absorbance at 235 nm in a UV spectrophotometer using H_2SO_4 solution as blank. The amount of PHB per millimetre of bacterial cells was determined by comparing absorbance readings with a standard crotonic acid curve.

EFFECT OF NUTRITIONAL AND PHYSIOCHEMICAL PARAMETERS

The effect of some nutritional parameters such as carbon sources (1%) (sucrose, galactose, starch and mannitol); nitrogen sources (0.5%) (potassium nitrate, ammonium nitrate, ammonium sulphate and ammonium chloride); mineral salts (0.25%) (calcium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and magnesium sulphate); a physiochemical parameter, agitation (150-300 rpm) on growth and PHB production of the selected strains was determined using one factor at a time (OFAT) method.

STATISTICAL ANALYSIS

The experiments were performed in triplicate and the representative average results were analysed statistically. The treatment effects were compared and the significant differences among replicates presented as Duncan's multiple range tests in the form of probability values (Duncan 1955).

RESULTS AND DISCUSSION

Even though several studies have shown that a number of bacterial strains, both Gram positive and Gram negative, produce PHB, the sheer diversity of the microbial world calls for continuous screening and identification of bacteria capable of utilizing cheap nutrient sources for production of large quantities of PHB. In this study, a total of twenty-one (21) bacterial strains namely; *Citrobacter* sp. ISO21, *Corynebacterium* sp. ISO10, *Bacillus* sp. (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 & 11), *Enterococcus* sp. (1, 2 & 3), *Vellionella* sp. (1 & 2), *Lactobacillus* sp. ISO15, *Yersinia* sp. ISO13 and *Micrococcus* sp. ISO8 were isolated from groundnut plant rhizosphere and subjected to morpho-physiological and biochemical characterization. Isolation and screening of potential bacterial strains from natural environment and improvisation of bioprocess parameters has been an extensive area of research in the field of PHB production (Israni & Shivakumar 2015). Figure 1 shows the percentage occurrence of different bacteria isolated from the soil samples. From the total (21) bacteria isolated, *Bacillus* sp. had the highest (54.4%) percentage frequency of occurrence.

The generic level identification of the bacterial isolates was based on micro-morphological (Table 1) and biochemical tests (Table 2). Preliminary screening of the 21 bacterial isolates for PHB production by Sudan-black staining method (Table 3) showed that 18 bacterial strains were potent producers of PHB based on the appearance of deep-stained cells when viewed under a microscope while the remaining three isolates showed negative reaction. The bacterial strains were further evaluated for PHB production by screening using submerged fermentation technique; quantification was done spectrophotometrically and by comparing the absorbance readings with a standard crotonic acid curve. In this case, all the isolates were positive for PHB production. Based on Sudan-black staining and spectrophotometric quantification, four isolates with maximum PHB producing potential were selected for further studies.

The result that all the bacterial isolates showed PHB production in submerged fermentation contrary to that of the Sudan staining method could be due to the fact that the fermentation method is non-specific in screening strains for PHB production (Sindhu et al. 2011). Four strains namely *Bacillus* sp.1 (4.899^{a} mg/mL), *Enterococcus* sp. (4.366^{b} mg/mL), *Bacillus* sp.2 (4.320^{c} mg/mL) and *Citrobacter* sp. (4.265^{d} mg/mL) which produced maximum amount of PHB were selected for further studies towards maximizing PHB production under a submerged fermentation process.

Figure 2(a) shows the effect of different carbon sources on the growth of the selected PHB-producing bacteria. Starch stimulated best growth in three of the selected isolates; *Bacillus* sp.2 (1.733^{a}), *Citrobacter* sp. (1.600^{a}) and *Bacillus* sp.1 (1.389^{a}), respectively, with the exception of *Enterococcus* sp. that had its best growth (1.371^{a}) stimulated by galactose and its least growth (1.290^{d}) stimulated by starch. All the sugars studied

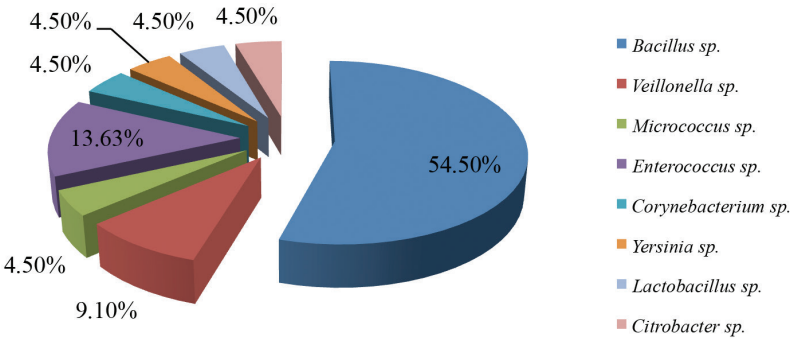

FIGURE 1. Percentage occurrence of bacterial isolates

TABLE 1. Morphological characterization of bacterial isolates

Isolate code	Morphological characterization of the isolates
ISO 1	Cream, raised, spherical, opaque, dry, undulate
ISO 2	Light brown, mucoid, rhizoid, raised, lobate, opaque
ISO 3	Cream, oblong, opaque, mucoid, entire, convex
ISO 4	Off white, transparent, mucoid, spherical, flat, undulate
ISO 5	Cream, circular, opaque, raised, mucoid, entire
ISO 6	Light brown, circular, opaque, raised, mucoid, entire
ISO 7	Cream, rhizoid, transparent, flat, mucoid
ISO 8	Cream, irregular, flat, undulate, mucoid, opaque
ISO 9	Off white, circular, dry, opaque, flat, entire
ISO 10	Off white, transparent, wrinkled, dry, flat, undulate
ISO 11	Off white, transparent, filamentous, flat, dry
ISO 12	Brown, mucoid, irregular, raised, opaque, lobate
ISO 13	Light brown, circular, mucoid, flat, opaque, lobate
ISO 14	Cream, spherical, mucoid, undulate, raised, opaque
ISO 15	Light brown, circular, opaque, raised, entire, rhizoid
ISO 16	Light brown, concentric, filamentous, transparent centre, mucoid, raised
ISO 17	Light brown, filamentous, mucoid, raised, lobate, opaque
ISO 18	Light brown, opaque, mucoid, undulate, rhizoid, raised
ISO 19	Cream, rhizoid, dry, dentate, transparent, flat
ISO 20	Cream, transparent, irregular, lobate, flat, dry
ISO 21	Cream, circular, raised mucoid, entire, opaque

TABLE 2. Biochemical and physiological characterization of bacterial isolates

Isolate code	Gram reaction	Morphology	Citrate	Catalase	Motility	Spore stain	Glucose	Galactose	Fructose	Maltose	Probable organisms
ISO 1	+ve	Rod	-ve	+ve	-ve	+ve	AG	A	AG	AG	*Bacillus* sp.1
ISO 2	+ve	Rod	-ve	+ve	-ve	+ve	A	-	-	-	*Bacillus* sp.4
ISO 3	+ve	Rod	-ve	+ve	-ve	+ve	AG	AG	AG	AG	*Bacillus* sp.10
ISO 4	-ve	Cocci	-ve	+ve	-ve	+ve	A	-	-	-	*Vellionella* sp.
ISO 5	+ve	Rod	-ve	+ve	-ve	+ve	AG	AG	A	AG	*Bacillus* sp.11
ISO 6	+ve	Rod	-ve	+ve	-ve	+ve	A	-	A	A	*Bacillus* sp.7
ISO 7	+ve	Rod	-ve	+ve	-ve	-ve	A	-	A	A	*Bacillus* sp.9
ISO 8	+ve	Cocci	-ve	+ve	+ve	+ve	AG	AG	A	AG	*Micrococcus* sp.
ISO9	+ve	Cocci	-ve	-ve	-ve	+ve	A	-	AG	AG	*Enterococcus* sp.
ISO10	+ve	Rod	-ve	+ve	-ve	-ve	A	AG	A	G	*Corynebacterium* sp.
ISO11	+ve	Rod	-ve	-ve	-ve	+ve	A	AG	A	G	*Bacillus* sp.8
ISO12	+ve	Cocci	+ve	-ve	-ve	+ve	AG	AG	A	A	*Enterococcus* sp.
ISO13	-ve	Cocci	+ve	+ve	-ve	-ve	A	-	A	AG	*Yersinia* sp.
ISO14	+ve	Rod	-ve	-ve	+ve	+ve	A	-	A	A	*Bacillus* sp.5
ISO15	+ve	Rod	-ve	-ve	-ve	-ve	AG	-	AG	AG	*Lactobacillus* sp.
ISO16	-ve	Cocci	-ve	-ve	+ve	+ve	A	-	A	A	*Vellionella* sp.
ISO17	+ve	Rod	+ve	-ve	+ve	+ve	AG	AG	A	A	*Bacillus* sp.3
ISO18	+ve	Cocci	-ve	-ve	-ve	-ve	AG	AG	AG	AG	*Enterococcus* sp.
ISO19	+ve	Rod	-ve	-ve	-ve	+ve	AG	AG	AG	AG	*Bacillus* sp.2
ISO20	+ve	Rod(cluster)	-ve	+ve	-ve	+ve	AG	AG	AG	AG	*Bacillus* sp.6
ISO21	-ve	Cocci	+ve	+ve	+ve	+ve	A	A	AG	A	*Citrobacter* sp.

KEY: A = Acid production; G = Gas production; AG = Acid and Gas production; +ve = positive; -ve = negative

(starch, sucrose, galactose and mannitol) supported good biomass growth (1.733[a], 1.402[b], 1.273[c] and 1.256[d]) of *Bacillus* sp.2, respectively. These selected bacterial isolates showed varied growth response to the different carbon sources tested. Berekaa and Al-Thawadi (2012) earlier reported that a strain of *Bacillus thuringiensis* IAM 12077 exhibited nutritional versatility in terms of varied growth and PHB production when tested on various carbon and nitrogen sources.

The effect of different carbon sources on PHB production by the selected bacterial isolates is shown in Figure 2(b). Maximum PHB accumulation was induced by mannitol (6.076[a] mg/mL) in *Bacillus* sp.1 while sucrose gave the best PHB production by *Bacillus* sp.2 (5.966[a] mg/mL), *Enterococcus* sp. (5.863[a] mg/mL) and *Citrobacter* sp. (5.796[a] mg/mL), respectively. Overall, the

TABLE 3. Screening of PHB-producing bacterial isolates

S/no	Probable organisms	Sudan-black staining	PHB Production (mg/mL)
1	***Bacillus* sp.1**	**+ve**	**4.899[a]**
2	*Bacillus* sp.4	+ve	3.811[i]
3	*Bacillus* sp.10	+ve	0.613[r]
4	*Vellionella* sp.	-ve	1.527[m]
5	*Bacillus* sp.11	+ve	0.536[t]
6	*Bacillus* sp.7	+ve	0.725[o]
7	*Bacillus* sp.9	+ve	0.640[q]
8	*Micrococcus* sp.	+ve	0.442[u]
9	*Enterococcus* sp.	-ve	0.610[s]
10	*Corynebacterium* sp.	+ve	4.048[g]
11	*Bacillus* sp.8	+ve	0.664[p]
12	***Enterococcus* sp.**	**+ve**	**4.366[b]**
13	*Yersinia* sp.	-ve	4.067[f]
14	*Bacillus* sp.5	+ve	3.436[j]
15	*Lactobacillus* sp.	+ve	4.009[h]
16	*Vellionella* sp.	+ve	1.210[n]
17	*Bacillus* sp.3	+ve	4.082[e]
18	*Enterococcus* sp.	+ve	2.686[l]
19	***Bacillus* sp.2**	**+ve**	**4.320[c]**
20	*Bacillus* sp.6	+ve	3.036[k]
21	***Citrobacter* sp.**	**+ve**	**4.265[d]**

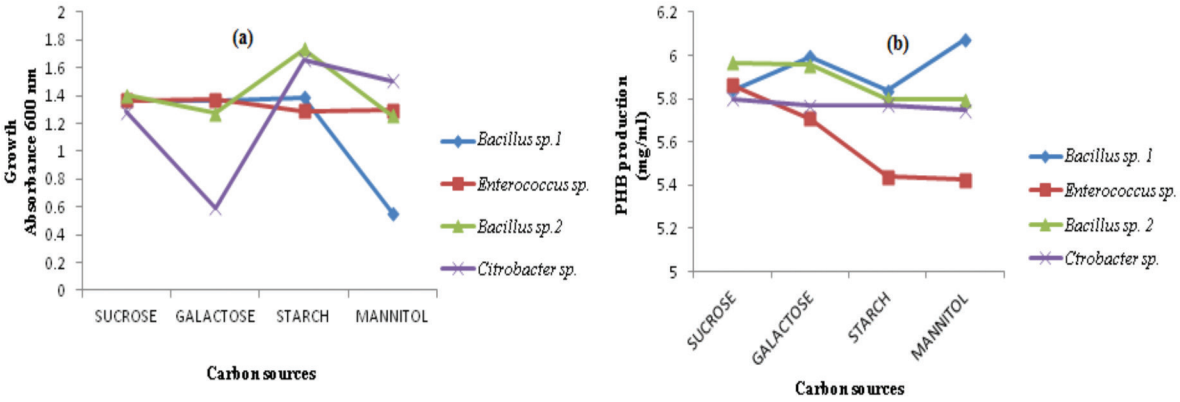

FIGURE 2. Effect of carbon sources on (a) growth and (b) PHB production by the selected bacterial isolates

different commercial carbon sources studied stimulated good PHB production by the selected bacterial isolates except starch which is a complex molecule. Starch is not easily utilized for effective PHB production because of its complexity, thus as the complexity of starch increases, PHB yield decreases (Israni & Shivakumar 2015). The results obtained in this study showed varying behavioural response to different sources of carbon by the selected organisms with respect to PHB accumulation. This is in accordance with the study of Belal (2013), where mannitol was found to be the best carbon source for *Rhizobium elti,* but in the case of *Pseudomonas stutzeri* isolated from rhizosphere soil and root noodles of bean plants; sucrose was found to induce the best PHB production by the strain. Carbon sources are critical since they serve three different functions that is, biomass synthesis, cell maintenance and PHB polymerization within the organism (Hungund et al. 2013). Moreover, the low yield of PHB observed in our findings could be attributed to the small size (25 mL) of the fermenter, since a large fermenter is expected to hold larger volumes of nutrient for microbial utilization. There is a linear relationship between the number of generation of cells (biomass) and the fermenter volume (Lonsane et al. 1991). That is, the larger the fermentation vessel holding the nutrient, the more the number of microbial cells produced, thus resulting in increased production of PHB.

The effect of different nitrogen sources on growth of the selected PHB-producing bacteria is shown in Figure 3(a). Bacterial biomass ranged from 1.560[a]-1.258[d], 1.554[a]-1.050[d], 1.542[a]-1.215[d] and 1.321[a]-1.267[d] for *Citrobacter* sp., *Bacillus* sp.2, *Enterococcus* sp. and *Bacillus* sp.1, respectively. Ammonium chloride (NH_4Cl)

stimulated best growth in *Citrobacter* sp. (1.560[a]) and *Bacillus* sp.2 (1.554[a]) while *Enterococcus* sp. (1.542[a]) and *Bacillus* sp.1 (1.321[a]) had best growth when ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) were utilized as source of nitrogen, respectively. This showed that the ability of bacteria to utilize different nitrogen sources substrates is variable and is independent of the nature of the substrate used (Belal 2013). Figure 3(b) shows the effect of nitrogen sources on PHB production by the selected bacterial isolates. Among the different nitrogen sources tested, KNO_3 was found to be the best for PHB production by *Citrobacter* sp. (5.728[a] mg/mL), *Bacillus* sp.1 (5.625[ab] mg/mL) and *Enterococcus* sp. (5.478[a] mg/mL) and *Bacillus* sp.2 (4.274[a] mg/mL) followed in order by NH_4NO_3 for *Citrobacter* sp. (5.670[b] mg/mL) and *Enterococcus* sp. (4.408[b] mg/mL), respectively, while the next promising nitrogen source for *Bacillus* sp.2 was ammonium sulphate $(NH_4)_2SO_4$ (4.247[b] mg/mL). The observation that KNO_3 stimulates the best biomass and PHB production in some of the isolates corroborates findings from a study conducted by Elsayed et al. (2013) on PHB production by a soil bacterial isolate, *Azomonas macrocytogenes*. NH_4Cl exhibited maximum positive effect on PHB production in this present study because it represents a readily utilizable nitrogen source for the process organisms, i.e. *Citrobacter* sp. and *Bacillus* sp.2. High PHB accumulation by strains utilizing ammonium (NH_4^+)-containing nitrogen source can be of advantage with respect to industrial applications of the strains, wherein ammonia containing waste liquids of various industries could be utilized for PHB production (Israni & Shivakumar 2015).

Figure 4(a) shows the effect of mineral sources on growth of the selected PHB-producing bacteria. Bacterial growth ranged from 1.930[a]-1.363[d], 1.868[a]-1.286[d], 1.615[a]-1.512[d] and 1.613[a]-1.524[a] for *Enterococcus* sp., *Citrobacter* sp., *Bacillus* sp.2 and *Bacillus* sp.1, respectively. Dipotassium hydrogen phosphate (K_2HPO_4) supported best growth in *Enterococcus* sp. (1.930[a]) and *Citrobacter* sp. (1.868[a]) while *Bacillus* sp.2 (1.615[a]) and *Bacillus* sp.1 (1.613[a]) had their best growth when magnesium sulphate ($MgSO_4.7H_2O$) was utilized as mineral source. This observation was in agreement with the work of Wei et al. (2011) where increased cell growth of

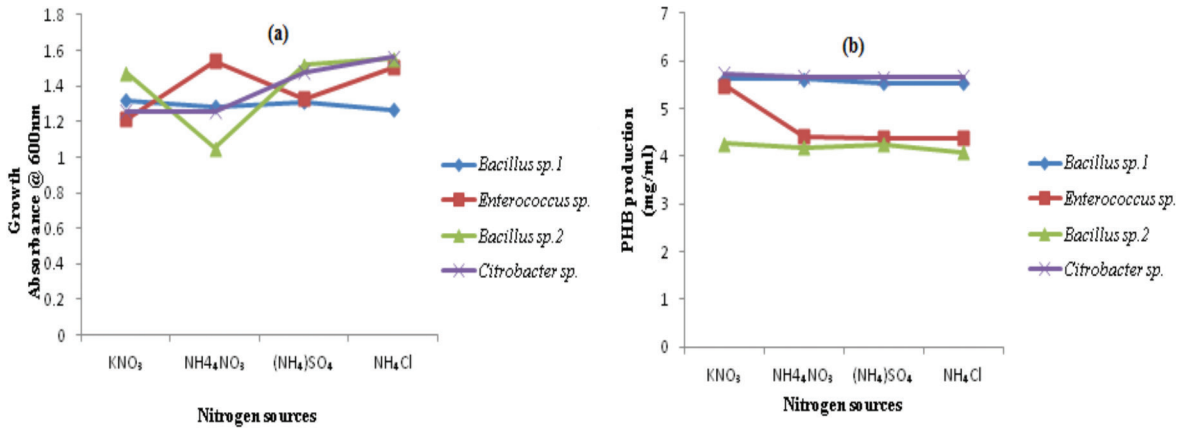

FIGURE 3. Effect of nitrogen sources on (a) growth and (b) PHB production by the selected bacterial isolates

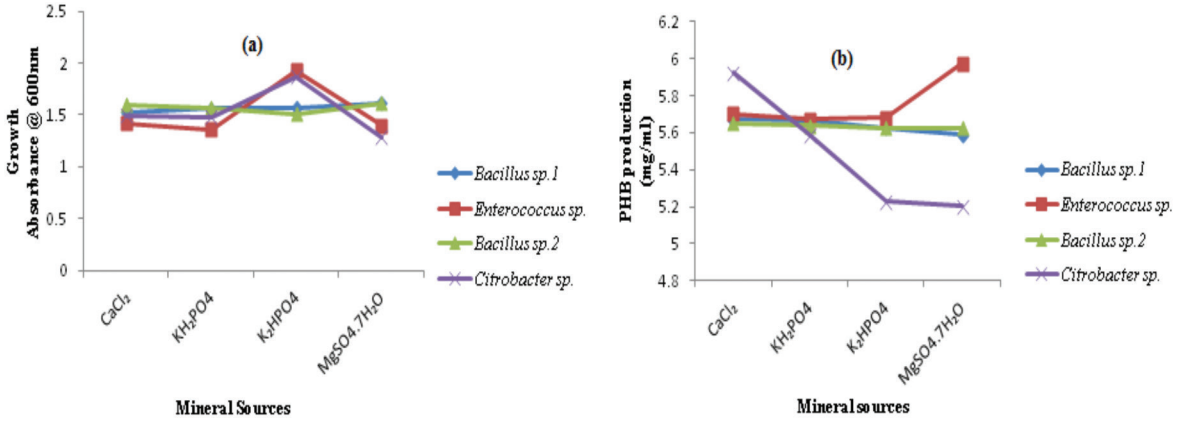

FIGURE 4. Effect of mineral salts on (a) growth and (b) PHB production by the selected bacterial isolates

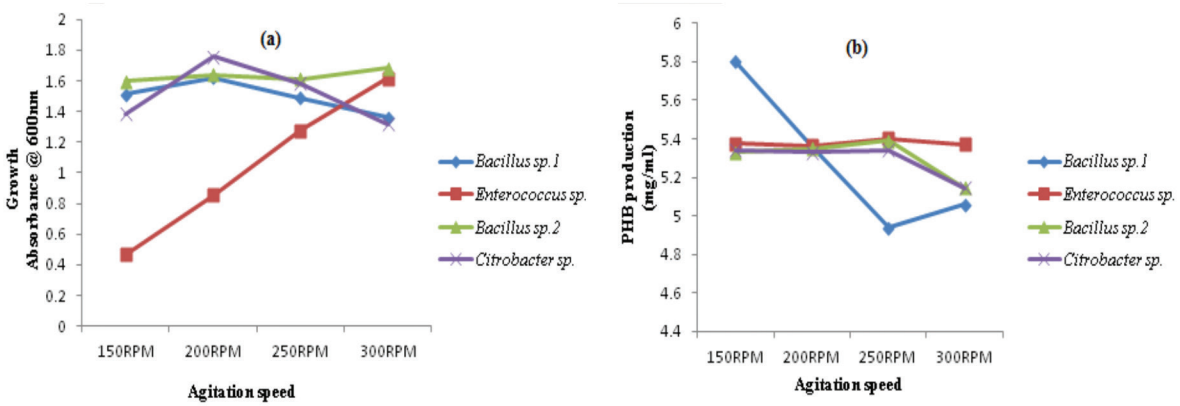

FIGURE 5. Effect of agitation speed on (a) growth and (b) PHB production by the selected bacterial isolates

Vibrio sp. BM1 was observed when the basal medium was supplemented with $MgSO_4.7H_2O$ and KH_2PO_4 singly and in combination. Mineral salts such as disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) and $MgSO_4.7H_2O$ are important for supporting bacterial life and as the critical material for synthesizing metabolites (Wei et al. 2011).

The effect of different mineral sources on PHB production by the selected bacterial isolates is shown in Figure 4(b). Calcium chloride ($CaCl_2$) stimulated optimum PHB production in *Citrobacter* sp. (5.924[a] mg/mL), *Bacillus* sp.1 (5.674[a] mg/mL) and *Bacillus* sp.2 (5.649[a] mg/mL) followed in order by KH_2PO_4, K_2HPO_4 and $MgSO_4.7H_2O$, respectively. These results were in conformity with that of Zahra et al. (2009) where they observed that $CaCl_2$ had the most significant effect on cell growth and PHB production of *Methylobacterium extorquens* DSMZ 1340 and also that KH_2PO_4 had a positive effect on PHB production by the isolate. The possible explanation for this effect is the important role of calcium and other trace metal ions in regulation of various enzymatic activities that catalyze the PHB biosynthetic pathway (Sasidharan et al. 2015).

Figure 5(a) shows the effect of different agitation speeds on growth of the selected bacterial isolates. The optimum agitation speeds for the growth of *Citrobacter* sp., *Bacillus* sp.2, *Bacillus* sp.1 and *Enterococcus* sp. were 200 rpm (1.760[a]), 300 rpm (1.684[a]), 200 rpm (1.620[a]) and 300 rpm (1.616[a]), respectively. Aeration of the culture during growth affects the accumulation of biopolymer and elevation of agitation rate apparently increased cell growth. Conversely, slower agitation speed may cause the possibility of cell aggregation, thus making the culture medium more heterogeneous. This eventually may cause decrease in cell growth hence affecting PHB production (Mohd-Zahari et al. 2012). The effect of different agitation speeds on PHB production by the selected bacterial isolates is shown in Figure 5(b). The optimum agitation speeds for PHB production by *Bacillus* sp.1, *Enterococcus* sp., *Bacillus* sp.2 and *Citrobacter* sp. were 150 rpm (5.802[a] mg/mL), 250 rpm (5.402[a] mg/mL), 250 rpm (5.390[a] mg/mL) and 250 rpm (5.341[a] mg/mL), respectively. Baei et al. (2010) also reported 250 rpm to be the maximum agitation speed for optimum PHB accumulation in *Azohydromonas lata* DSMZ 1123 and *Pseudomonas* sp. On the other hand, a low agitation speed (175 rpm) was also reported to give maximum PHB yield (7.48 g/L) by *Cupriavidus necator* (Aramvash et al. 2015). The general trend shown by the selected bacterial isolates is that, as the agitation speed increases the biomass growth and PHB production also increases. However, higher than the optimum speed of the various selected bacterial isolates, cell growth and PHB production declines. The results obtained in the present study was in agreement with the work of Mohd-Zahari et al. (2012) where similar trend was reported for agitation effect on bacterial cell biomass and P(3HB) accumulation. In conclusion, the present study showed potential of the selected bacterial isolates to produce high quality PHB and that its production was greatly influenced by nutritional parameters such as carbon, nitrogen and mineral sources as well as other physiochemical parameters. It was also noteworthy that the PHB produced by the selected strains was growth-associated. The need to explore other environment harbouring microbial strains with high PHB-producing potential was paramount to the discovery of bioplastics with improved properties for potential industrial application.

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