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Production of polyhydroxyalkanoates (PHAs) by microorganism from contaminated soil, industrial wastewater and domestic wastewater

Kanokwan Nantha[1], Pilanee Vaithanomsat[2,*], Antika Boondaeng[2], Chanaporn Trakunjae[2], Sawitri Chuntranuluck[1]

[1] Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok 10900, Thailand
[2] Enzyme Technology and Waste Management Research Unit, Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Kasetsart University, Chatuchak, Bangkok, 10900, Thailand
*E-mail: aappln@ku.ac.th

Abstract

Polyhydroxyalkanoates (PHAs) is a bio-polymer accumulated in a bacterial cell. PHA is a potential substitute in fuel industry, medical industry and material industry such as biofuel, surgical pins and latex. This work is to isolate PHA producing bacteria from sources, including contaminated soil, industrial wastewater and domestic wastewater in Bangkok Thailand. Total 250 strains screening for PHA production using Sudan black B, Nile blue A and Nile red staining methods. After, preliminary staining 9 isolates exhibiting high PHA production were confirm by High Pressure Liquid Chromatography (HPLC). The Strain KU G15 was selected for further analysis due to its high PHA production at 11.69 % by cell dry weight. Effect of carbon sources and nitrogen sources on growth and PHA production of strain KU G15 were investigated. Strain KU G15 was grown on mineral salt medium supplemented with different carbon sources (glycerol, palm oil, starch, glucose, sucrose, fructose, maltose, and lactose) and nitrogen sources (tryptone, peptone, yeast extract, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $NaNO_3$ and KNO_3). The best result was obtained when using 30 g/l glycerol and 0.5 g/l KNO_3 as carbon and nitrogen sources, respectively, demonstrating the accumulation of PHA up to 32.07 % and 34.87 %, respectively. The central composite design (CCD) was further applied to define the optimal production condition. Based on statistical analysis, maximal PHA production was reached using the optimal medium composition.

Introduction

Now-a-days, non-biodegradable waste materials have been a growing concern of scientific researchers in response to the problems in public health and environmental issues. Several researches and development on biodegradable plastics suggesting that bacteria, archaea, and in few eukaryotes such as yeasts and fungi can produce bioplastic in the form of polyhydroxyalkanoates (PHAs). PHAs are usually the energy storage inclusion synthesized intracellularly in the presence of excess carbon with limiting concentration of nitrogen, phosphorus, sulphur or oxygen essential for growth. They are natural, renewable and biocompatible biopolymers which can be used as substrates for plastic materials with properties similar to petrochemical or synthetic plastics. The family of PHA includes several polymeric esters such as polyhydroxybutyrates (PHB), polyhydroxy butyrate-co-hydroxyvalerates (PHBV), polyhydroxybutyrate-co- hydroxyhexano ate (PHBHx) and polyhydroxybu tyrate-co-hydroxyoctonoate (PHBO) [1].

The aim of this study was to isolate the new microbial strain which can produce PHA from oil contaminated soil and waste water sample in Thailand. The identification based on genetic techniques and statistical evaluation of appropriate carbon source and nitrogen source were tested in this study.

Methodology

Collection of samples

Contaminated soils and wastewater samples were randomly collected using a sterile 50 mL conical centrifuge tube from the different areas in Bangkok Metropolitan Region, Thailand, i.e., central canteen and wastewater treatment pond in Kasetsart University, wastewater treatment pond in the Patum Vegetable Oil and Wan Thai Industry Factory. A total of 35 soil and wastewater samples were kept on ice while being transported back to the laboratory.

Isolation of strains

The contaminated soil and wastewater samples were spread on nutrient agar (NA) (Merck, USA) after serial dilution upon reaching laboratory. All cultures were incubated at room temperature (30-37°C) for 3 days. Colonies were subcultured on NA until pure cultures were obtained. Culture stocks were kept on nutrient agar slant and kept at 4 °C for further studies.

Screening for PHAs-producing microorganisms

All different collected isolates were screened for PHA production. The first method, Sudan Black B staining [2], a lipophilic staining reagent was used to stain the lipid granules, helping in differentiating the PHAs producers and non-producers. All isolates were incubated on mineral salt medium (MSM) agar, which consisted 2.8 g/l KH_2PO_4, 4.16 g/l $Na_2HPO_4.2H_2O$, 0.5 g/l NH_4Cl, 0.25g/l, $MgSO_4.7H_2O$, 30 g/l glucose [3] and 1 ml/L of trace element (26.73 g/l $CaCl_2.7H_2O$, 1.3 g/l $ZnSO_4.7H_2O$, 0.6 g/l $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.2 g/l $FeSO_4$. $7H_2O$ and 0.03 g/l $CuSO_4$ $5H_2O$) at room temperature for 72 h. After that, the samples were heat fixed and stained with Sudan Black B Solution for 10 minutes; excess staining reagent was clarified using Xylene and the stained samples were blot dried and countered with 0.5 % aqueous Safranine. The samples were then observed under the microscope oil immersion at 1000x magnification. The dark blue colored granules in the cells were taken as positive for PHAs production. Nile blue A and Nile red staining were carried out as a second screening method to determine the PHAs accumulating ability of the isolated microbial strains.[4] The medium prepared for Nile red screening consisted of MSM (as described above) supplemented with 0.5 mg/ml of Nile blue A and Nile red. All isolated pure bacterial cultures were streaked on MSM agar supplemented with Nile blue A and Nile red to screen for PHA accumulating bacterial strains. All bacterial cultures were incubated at room temperature for 3 days. The isolates showing the bright blue and orange fluorescence upon irradiation with UV light after Nile blue A and Nile red staining were selected as PHAs accumulators and their fluorescence intensity increased with the increase in PHAs accumulation contents.

Identification of PHAs accumulating microbial strain

Isolation of genomic DNA was carried out by boiling the selected cells with lysis buffer according to the methods of [5] with slight modification. A loopful of yeast cells was transferred to 1.5 ml Eppendorf tube. The 100 µl of lysis buffer was added. Cell suspensions were boiled in water bath or metal block bath for 15 min. After boiling, 100 µl of 2.5 M potassium acetate (pH 7.5) was added and placed on ice for 1 h, and centrifuged at 14,000 rpm for 5 min. Supernatant was extracted twice with 100 µl of chloroform:isoamyl alcohol (24:1 v/v). DNA was precipitated with isopropanol, placed at 20°C for 10 min and centrifuged at

15,000 rpm for 15 min. DNA pellet was rinsed with 70% and 90% ethanol and then dried up (15-30 min at room temperature). The dried DNA was dissolved in 30 µl milli Q water.

The divergent D1/D2 domain of 26S rDNA was amplified with primers NL-1 (5'- GCA TAT CAA TAAGCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') [6]. Amplification was carried out in 100 µl reaction mixture conditioning 100 ng of genomic DNA, 2.5 U of Taq polymerase, 20 mM of each dNTP, 40 mM of each primer, 10 mM Tris-HCl and 1.5 Mm $MgCl_2$. The reaction was pre-denatured at 94°C for 5 min, then repeated for 30 PCR cycles at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2.5 min and then followed by the final extension at 72°C for 10 min. The amplified DNA was purified with QIAquick PCR Purification Kit according to the manufacturer's instruction. Visualization of the purified amplified DNA was performed by electrophoresis using 0.8% agarose gel in 1X TBE buffer and stained with ethidium bromide ($8x10^{-5}$ µg/ml) and observed under UV illuminator. The nucleotide sequences of D1/D2 domain of 26S rDNA were directly determined using PCR products according to [6] with slight modification. Cycle sequencing of D1/D2 domain was employed with forward primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAAAAG-3'), and reverse primer, NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'), by ABI PrismTM BigDyeTM Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA) according to the manufacturer's instruction. Sequences were assembled and edited with the Sequencher program (version 3.1). Multiple sequence alignment was performed by using CLUSTALX. The multiple alignment output from CLUSTAL_X was imported and manually edited with the BioEdit (version 7.2.5). Pairwise sequence comparisons were expressed as the percentage of the total number of nucleotide differences divided by the total number of positions. Phylogenetic analysis was performed by using the phylogeny inference package, MEGA (version7)

Effect of carbon source on PHAs production

The selected isolate was grown in 250 ml Erlenmeyer flasks containing 100 ml MSM medium supplemented with different carbon sources i.e., glycerol, palm oil, starch, glucose, sucrose, fructose, maltose and lactose at 3% (w/v) for 72 h with shaking of 200 rpm at room temperature. Samples were taken for PHAs analysis and biomass; the best carbon source was selected for further studies.

Effect of nitrogen source on PHAs production

The selected isolate was grown in 250 ml Erlenmeyer flasks containing 100 ml MSM medium with the best carbon source. Different nitrogen sources were varied, including tryptone, peptone, yeast extract, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $NaNO_3$ and KNO_3 for 72 h with shaking of 200 rpm at room temperature. Samples were taken for PHAs analysis and biomass; the best nitrogen source was selected for further studies.

Analysis techniques

Determination of biomass was performed according to [7]. The 10% (v/v) initial culture was added in 100 ml MSM culture broth. The flask was incubated for 48h with shaking of 200 rpm at room temperature. Then, the cells were harvested by centrifuge at 8000 rpm at 4°C for 10 min. The obtained cell pellet was then washed twice with distilled water by resuspension and centrifugation as above. The cells were dried at 95°C for 24 h in a hot air oven then cooled down in desiccators.

Determination of PHAs content was performed according to [8]. The cell samples were and digested in 1 ml concentrated H_2SO_4 at 100°C for 30 min. The tubes were cooled on ice, and diluted with 4 ml of 0.014 N H_2SO_4 was added with rapid mixing. Before analysis by HPLC, samples were diluted an additional 5-to 100-folds of 0.014 N H_2SO_4 containing 0.8 mg of adipic acid per ml as an internal standard. High Pressure Liquid Chromatography (HPLC)

(Shimadzu, Japan) was connected with Aminex PHX-87H ion-exclusion column. The 20 µl of samples were injected and then eluted with 0.014 N H_2SO_4 at a flow rate of 0.6 ml/min at 60°C absorbance at 210 nm. The amount of crotonic acid produced from PHAs was calculated from the regression equation derived from crotonic acid standards (Sigma-Aldrich).

Results and Discussion

Isolation and Screening of PHAs-producing microorganisms

A total of 35 samples were collected from soil and waste water samples in Bangkok, Thailand. Different isolates representing different colony morphologies were picked-up randomly from the NA plates. A total of 250 different types of colonies with positive PHAs production were picked up from the MSM plates. After Sudan black B staining, 84 isolates with distinct black granules (Figure 1.), indicating PHAs accumulation in cells, were picked up for further staining with Nile blue A and Nile red, respectively (Figure 2). Nine isolates with positive PHAs accumulation, showing as bright blue and orange fluorescence cells under UV light after Nile blue A and Nile red staining, were selected for PHAs quantitation.

The 9 PHAs-positive isolates were grown in 50 mL of MSM medium using excess glucose as the carbon source and limiting NH_4Cl as the nitrogen source. Among the 9 isolates, isolate KU G15 showed the highest PHAs content of 11.69%CDW with 0.84±0.06 g/L of biomass. Whereas, the lowest yield of PHAs (4.08%CDW) was obtained by isolate KU 90-1A (Table 1).

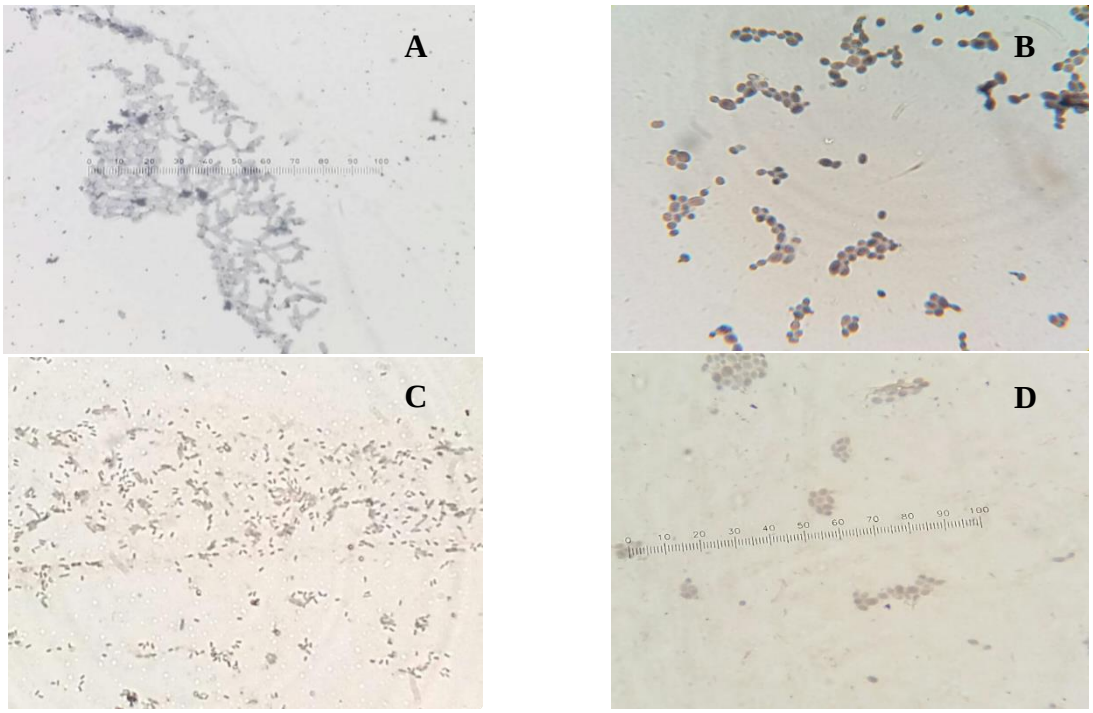

Figure 1. Sudan black B staining of PHAs granules (black inclusion) in the cells KU G15 (1A), KU H5 (1B), KU 90-6A (1C), and KU 53A (1D) under 1000X magnification observation.

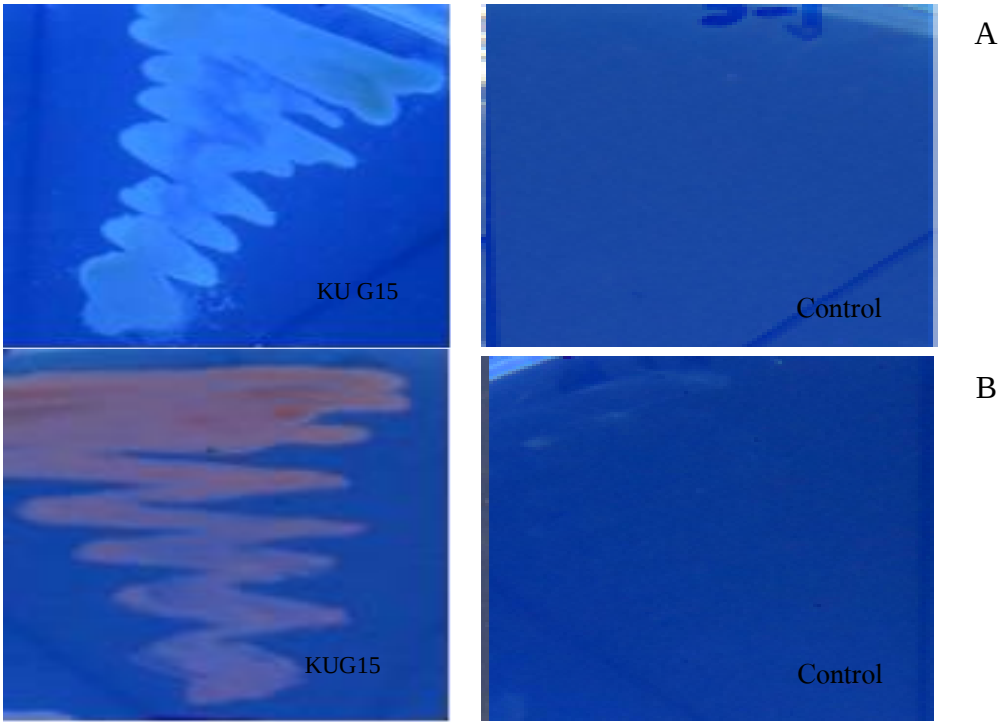

Figure 2. Nile blue A (2A) and (2B) Nile red staining of isolates KU G15 under UV light.

Table 1. PHAs content (%CDW) of 9 isolates grown in MSM medium at room temperature with shaking at 180 rpm for 72h.

Isolate	CDW (g/l)	PHA content (%CDW)
KU1-6	1.59±0.16	5.81±0.36
KU 42C	0.33±0.02	10.82±0.02
KU G15	0.84±0.06	11.69±0.85
KU 33B1	0.36±0.01	8.34±0.59
KU 53A	2.64±0.20	5.54±0.58
KU 90-1A	0.35±0.02	4.08±0.69
KU 90-6A	1.14±0.04	7.11±0.35
KU 10K-5	0.81±0.02	8.12±0.03
KU H5	1.48±0.05	9.96±0.59

Identification of PHAs-producing strains

Analysis of the 26S rDNA gene sequences of isolate KU G15 was performed using NCBI-BLAST (National centre for Biotechnology Information http://www.ncbi.nml.nih.gov). BLASTn result for the 26S rDNA gene sequences of isolates KU G15 showed 100% sequence identities to *Rhodotorula mucilaginosa* CBS 316T (NG_055716). A neighbor-joining phylogenetic tree was constructed using the 26S rDNA gene sequence of isolate KU G15 and other closely related yeast strains according to the GenBank database, which were members from the genus *Rhodotorula* and genus *Rhodosporidium*. From the 26S rDNA gene phylogenetic analysis, isolate KU G15 shared the same latest common origin and evolutionary lineage with *R. mucilaginosa* (Figure. 3), indicating that isolate KU G15 could be classified under the genus *Rhodotorula* and hence was named as *Rhodotorula mucilaginosa* KU G15.

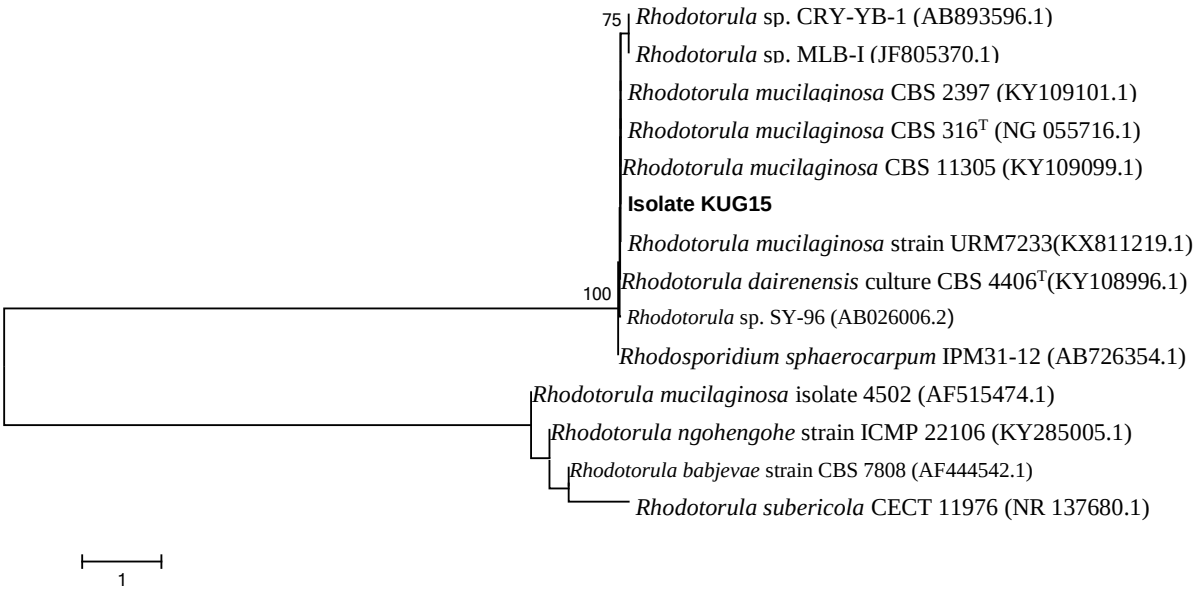

Figure 3. The evolutionary history was inferred using the Neighbor-Joining method [9]. The optimal tree with the sum of branch length = 15.73051165 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [10]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [11] and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 461 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. [12]

Effect of carbon sources on PHAs-production

To increase the yield of PHAs, various carbon sources (3% w/v) such as glycerol, palm oil, starch, glucose, sucrose, fructose, maltose, and lactose were added to the MSM medium with the inoculum of 2% (v/v) and cultivation for 72 h with shaking of 200 rpm at room temperature (30°C). The carbon source tested in this study clearly affected PHAs production by *Rhodotorula mucilaginosa* KU G15 as shown in Figure 4. Maximum PHAs accumulation at 32.07% CDW was detected when 30 g/l glycerol was used. Various carbon sources also influenced the cell dry weight. Similar results were reported by Chengium Zhu in 2009[13], when poly-3-hydroxybutyrate (PHB) was maximally synthesized in the glycerol-containing media.

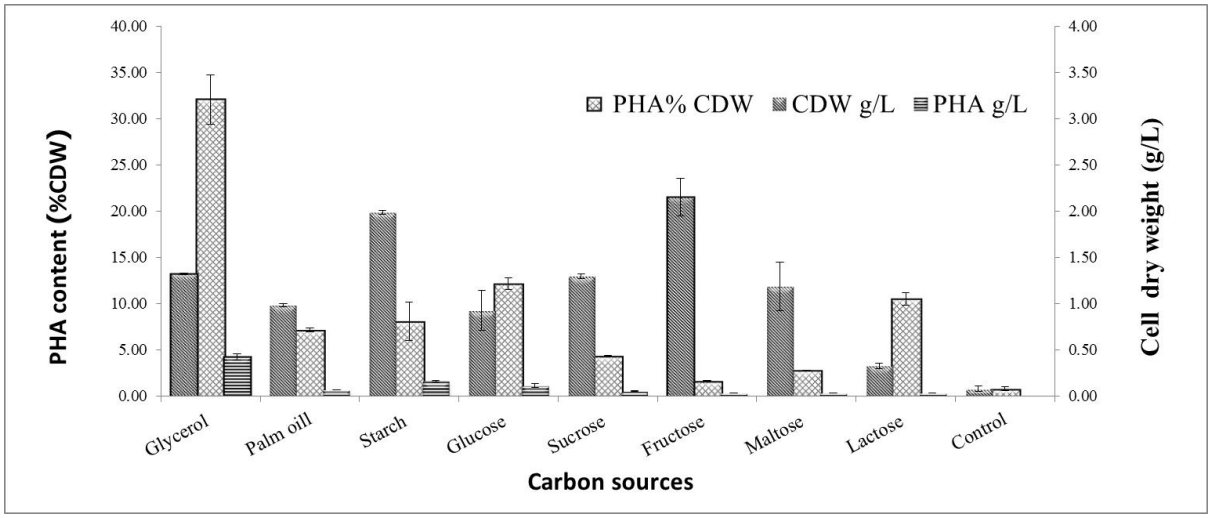

Figure 4. Effect of various carbon sources on PHAs content (%CDW).

Effect of nitrogen sources on PHAs production

Nitrogen is an essential factor for PHAs accumulation in microbial strains under stressed conditions. Various biologically active complexes, organic and inorganic nitrogen sources, such as tryptone, peptone, yeast extract, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $NaNO_3$ and KNO_3, were tested at an equal amount (0.5 g/l) and cultivation for 72 h with shaking of 200 rpm at room temperature (30ºC) (Figure 5). It was found that, maximum PHAs accumulation of 34.87 % DCW was obtained when KNO_3 was used as a nitrogen source thus it was served as a nitrogen source for further experiments. The effect of different nitrogen and carbon sources on PHAs accumulation in microorganisms was previously reported. Mercan (2002) [14] investigated the PHAs production in 2 strains of *Rhizobium* sp. They noted that the strains produced less PHAs in yeast extract mannitol (YEM) broth media with different carbon (glucose, sucrose, arabinose) and nitrogen (L-cysteine, L-glycine, DL-tryptophan, proteose peptone, potassium nitrate) sources, while the highest level of PHAs accumulation was observed in the media with L-cysteine, L-glycine.

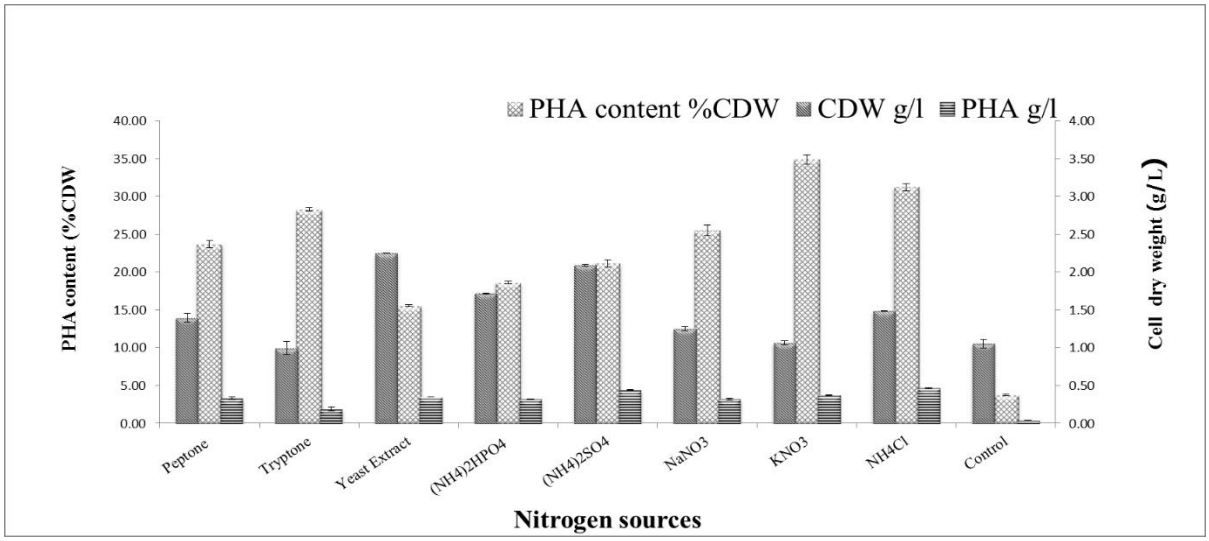

Figure 5. Effect of various nitrogen sources on PHAs content (%CDW).

Conclusion

In present study, 250 different microbial isolates were obtained from oil contaminated soil and waste water samples. The highest amount of PHAs production (34.87 %CDW) was obtained from the isolate *Rhodotorula mucilaginosa* KU G15, identified as *Rhodotorula mucilaginosa*, using the medium consisted of 30 g/l glycerol, 0.5 g/l KNO_3, 4.16 g/l $Na_2HPO_4.2H_2O$, 2.8 g/l KH_2PO_4, 0.25 g/l $MgSO_4.7H_2O$ and 1 ml of g/l trace element solution. The ability of *R. mucilaginosa* KU G15 for the high production of PHAs could be the starting point to achieve cost-effective production of the biodegradable polymer, which is a great hindrance in the commercial use of PHAs. Additionally, to enhance the PHAs production by this strain, the medium composition and key physical parameters are required for further studies.

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