Original article

Comparison of intracellular polyhydroxybutyrate granules formation between different bacterial cell subpopulations by flow cytometry

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Abstract

Introduction and objective: Intracellular polyhydroxyalkanoate granules (PHA) are biodegradable polyesters and produce by different kind of prokaryotic microorganisms. Polyhydroxybutyrate (PHB) is one of the PHA polyesters and consists of hydroxybutyrate unit. PHB has a wide range of applications in pharmacy and drug delivery systems. The aims of this study were PHB production and determination of granules distribution in different subpopulations of single genus by Nile red staining method and flow cytometry.

Materials and methods: Bacterial PHB producers were screened by viable colony staining method. Kinetic study or secondary screening of strains was performed by fluorometric and photometric methods. Also distribution of PHB granules in different subpopulations of single genus of *Ralstonia* and *Sinorhizobium* had been determined by flow cytometry.

Results: Different genera of bacteria were isolated and selected for PHB production. Among all studied strains, *Ralstonia* (L1) and *Sinorhizobium* (AR2) have the highest ability for granule formation. Flow cytometry distinguished four subpopulations (QA1, QA2, QA3 and QA4) with different PHB content and side scatter parameter in both strains at Mid-log phase.

Conclusion: These heterogeneous subpopulations have diverse characteristics related to granule formation and multiplication activity. Flow cytometry showed each individual subpopulation has specific cell size, complexity and granularity related to cell biochemical and physiological states.

Significance and impact of the study: These strains are not pathogen and have been isolated from plant roots and soil. They are appropriate to get high PHB for drug delivery applications.

Keywords: Polyhydroxyalkanoate; Intracellular PHB Granules; *Ralstonia*; *Sinorhizobium*; Flow cytometry

Introduction

Different genera of prokaryotic microorganisms (eubacteria and archea) synthesize polyhydroxyalkanoates (PHAs) as water insoluble intracellular particles [1,2]. Production of polyester particles is induced when carbon source is in excess quantity or growth conditions have been imbalanced by declining nitrogen source or other factors [3-9]. Once the limiting nutrients are available, these compounds are degraded by intracellular enzymes and carbon and energy supply [10-12].

Polyhydroxyalkanoates (PHA) polymers are biodegradable when exposed to environmental conditions and thereby have many applications in agriculture, food industry, medicine and pharmacy [13-16]. The culture conditions have main effects to induce PHA production by bacteria. Moreover, different genera or different subpopulations of single genus do not have the same physiological response when exposed to the same culture conditions [17].

Cell analyzers or cytometry techniques are suitable for population analysis and intracellular granules formation [18]. Staining methods based on Nile red in combination with the above techniques can be used to identify the presence of PHAs polymer in the bacterial populations and subpopulations [19-21]. These methods are fast and very sensitive in the discovery of polyesters. Also real-time polyhydroxybutyrate production and content of single cell can be assayed by flow cytometry [22]. Flow cytometry is a non-invasive technique for quantitative measurement of intracellular PHA particles based on Nile red excitation at 488nm and fluorescence emmition at 585nm.

Flow cytometry analyzes the large number of single cells for several parameters simultaneously and counting the high number of cells in each sample provides statistical data about the granule formation in bacterial populations [19-22]. Fluorescence intensity of polyester granules stained with Nile red, forward and side scatters of the cells provide useful information about starting time of granules formation. Also, cell contents or quantity of polymer and kind of cell physiological response to culture conditions can be determined by this method. In this study different PHA producers were isolated and screened. Among the isolates, subpopulations of two genera, *Ralstonia* and *Sinorhizobium*, were analyzed at different stages of growth for PHA granule formation using flow cytometry. There are only few study to use flow cytometry to detect PHA.

Materials and methods

Primary screening of polyhydroxybutyrate (PHB) producers

The PHA accumulating bacteria were detected by a viable colony staining method. Briefly, portion of Nile red (Sigma, USA) (0.25mg NR per 1ml DMSO) was added to sterile medium with proper carbon source to a final concentration of 0.5µg per 1ml of culture medium. For solidified medium, 2% agar was used. Detection of PHA production was made by exposition of cultivated plates to UV (312nm) after 72h of incubation. Positive strains that could produced PHA, were detected by their orange fluorescent [23].

Secondary screening of PHB producers

Isolated bacteria from primary screening method were cultivated in proper broth media and shaker incubated at 30°C and 150rpm. Sampling was performed every hour. 1ml of culture medium was centrifuged and pellet resuspended in PBS and followed by centrifugation, then ethanol 35% was added for fixation of samples at room temperature for 15mins. Also biomass of cells was centrifuged and the optical

density was adjusted to 0.5 McFarland by UV/vis spectrophotometer. For staining the PHA in concentrated cells, 20µl of Nile red solution (1mg Nile red per 1ml DMSO) was added to the biomass to give a final concentration of 20µg/ml in 1ml cell suspension and incubated for 30mins. This suspension was used for flow cytometry analysis. Flow cytometry analysis was performed using a partec PAS system with an argon ion laser. Nile red was excited at 488nm and emitted fluorescent at 585nm collected in the FL_2 channel. Emitted fluorescence intensities of PHB granules were used in the analysis. 30,000 cells were analyzed in each sample [21].

Purification and chemical determination of PHA granule

The overnight culture of bacteria (*Ralstonia* L1 and *Sinorhizobium* AR2) in nutrient agar (Merck, Germany) were used for preparing the McFarland turbidity (0.5) as inoculate (5%v/v) to 100ml broth mediums in 250ml enclosed 0.5g NH_4Cl, 1g K_2HPO_4, 2g KH_2PO_4, 0.5g NaCl, 0.5g $MgSO_4$, $7H_2O$, 0.015g $CaCl_2$, 10g glucose or sucrose and 0.5g yeast extract for culture of *Ralstoni* and *Sinorhizobium* contained per liter. One ml of broth was centrifuged and pellet was resuspended in a one ml of PBS and centrifuged again. Cell pellet was disrupted with incubation in triton X-100 solution (1%) for 30mins at room temperature.

Cell suspension was centrifuged and washed with PBS buffer. 1ml of alkaline sodium hypochlorite solution was added to the pellet and incubated at 30°C for 1h. After incubation, the solution was centrifuged and sediment was washed by distilled water and alcohol for several times to remove the impurities. The washed white powder dried and 1ml concentrated sulfuric acid were added and incubated at 100°C in a water bath for 10min. The absorbance of crotonic acid was determined at 235nm against the sulfuric acid as blank after cooling. The category of PHA analyzed with HPLC and calibration line was determined by using pure PHB [24].

Biomass and cell growth determination

To measure biomass, 5-10ml of broth culture as centrifuged, resuspended in 10ml PBS and centrifuged again. The pellet was transferred to a pre-weight peteri dishes and incubated at 105°C to reach constant weight. Ammonium was determined by Nessler reagent and compared with calibration line of ammonium chloride.

Flow cytometry analysis

The cells were grown in broth medium for 24h, then harvested by centrifugation and cell pellet was incubated in above culture medium minus carbon source for 24h. Inoculum seed prepared by centrifugation and cell pellet washed with phosphate buffer saline (pH=7.0) and incubated in culture plus proper carbon source. Samples were prepared by centrifugation and washing in intervals. Cell pellets were preserved in 1ml of NaN_3 solution (10% in PBS) in 4°C until staining procedure. Cell staining procedure performed by modified method of Muller *et al*. [25].

In brief, preserved samples were centrifuged for 5mins in 5000rpm, supernatant was discarded and pellet was washed with PBS three times. Ethanol 35% was used for fixation of samples at room temperature for 15mins. Then the sample centrifuged again and with PBS the optical density of cell biomass adjusted to 0.5 according to McFarland turbidity. For staining the cells, 20µl of Nile red solution (1mg Nile red per 1ml DMSO) was added to test tube to give a final concentration of 20µg/ml in 1ml cell suspension and incubated for 30mins. This suspension was used for flow cytometry analysis. Side scatter signal Vs FL_2 parameter (Emitted

fluorescence intensity of Nile red stained granules) were used in the analysis [21,25].

Results

There are so many PHA polymers which are produced from many microorganisms. But industrial unit are produced from *Alcaligenes uropea*. PHA which can be used as drug delivery and biodegradation plastic materials could have different characteristics due to the length of polymer and their monomer unit. PHB is one of this polymers included of polyhydroxy butyrate unit. In this work for the first time in Iran *Ralstoni* has been isolated and the production of this polymer is detected by flow cytometry and compared with other PHA producer. Different genera of bacteria were isolated from activated sludge, soil and water samples.

Bacterial colonies were cultivated on mineral salt medium agar (MSM agar) containing Nile red with proper carbon source. The Nile red dye emitted orange fluorescence after binding to the intracellular polymer granules that were exposed to ultraviolet light. Six isolates showed orange fluorescence compared with *Ralstonia pickettii* AR1 which is positive PHA producer (Table 1).

Table 1: Orange fluorescence emitted by different strains cultured on MSM agar containing Nile red

Strains	Orange fluorescent observed by eye
Rhizobium 4R	++++
Azotobacter A1	+++
Rhizobium sin1	+++
Sinorhizobium AR2	+++
Ralstonia L1	+++
Ralstonia pickettii AR1 (positive control)	++

Several different species were screened by viable colony staining method. *Azotobacter*, *Ralstonia*, *Pseudomonas* and *Sinorhizobium* showed strong orange fluorescence under ultraviolet light higher than *R. pickettii* AR1 as a positive control. The purified PHA is identified as PHB by HPLC. The data is not shown here. Qualitative assessment of PHB granules formation was performed between selected strains by spectrofluorometrically determination of relative fluorescence intensity (FI) by flow cytometry in 12h interval (Fig. 1).

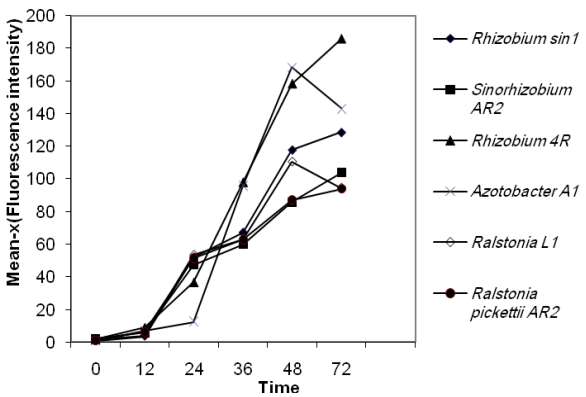

Fig. 1: Fluorescence intensities (FI) emitted by Nile red stained intracellular PHA granules assayed by flow cytometry every 12 h. FI is correlated to cellular PHA content

The kinetics of granule formation among the isolates was compared. The relative fluorescence intensity was high in *Sinorhizobium* AR2 after 72h. Two different strain, *Ralstonia* L1 and *Sinorhizobium* AR2 were selected for further analysis by flow cytometry. PHB accumulation in *Ralstonia L1* and *Sinorhizobium AR2* was determined spectrophotometrically by chemical method (Figs. 2, 3). Chemical analysis confirmed that strains without orange fluorescence spots do not have any intracellular PHB particles.

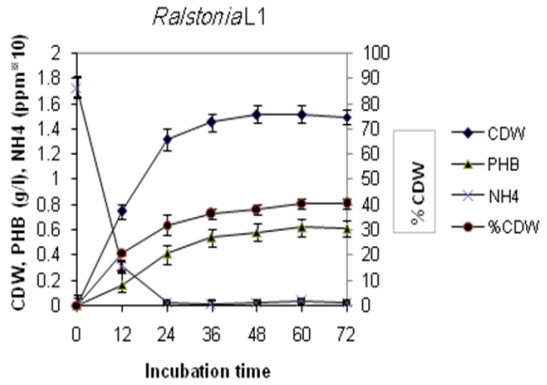

Fig. 2: Time profile analysis of PHA concentration (▲) and content or % cell dry weight (●) by *Ralstonia* L1 in shake flask at 30C and 150rpm. Intracellular PHB granule formed at log phase and stopped at stationary phase. Nitrogen source or NH_4 (×) reach to limiting level at 12h

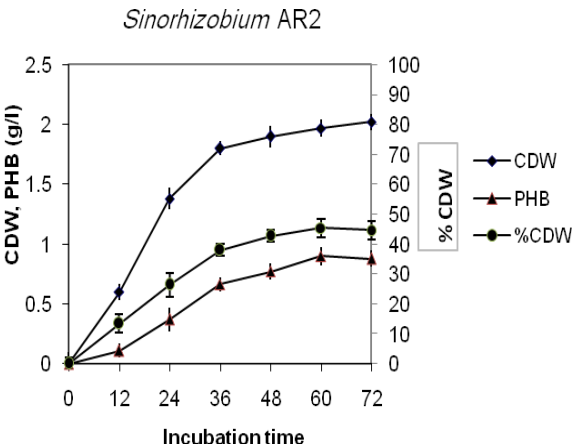

Fig. 3: Time profile analysis of PHA concentration (▲) and content or % cell dry weight (●) by *Sinorhizobium* AR2 in shake flask at 30C and 150rpm. Stationary phase initiated at 36h and cell proliferation was stopped but PHB production continued for 72h

Ralstonia L1 and *Sinorhizobium* AR2 were selected for flow cytometry analysis. Cell dry weight and polyester accumulation was assessed for these strains in broth medium. These parameters were increased rapidly during the exponential growth of *Ralstonia* L1 (Fig. 2). Rapid decline in NH_4 level was observed during mid-log growth phase. The PHB content as 36% cell dry weight (CDW) was obtained at 36th h (at early stationary phase). Accumulation was almost stopped during stationary phase. The PHB cell content levels of 36-40% CDW were obtained during stationary phase (36-72h). PHB production rate slowed down as well as growth rate and remained steady at stationary stage.

PHB concentration was 0.605g/l with PHB content of 40.26% CDW at 72h. PHB production was initiated in *Sinorhizobium* AR2 at 12th h of incubation (exponential growth phase). PHB production continued during stationary phase unlike *Ralstonia* L1 and reach to 0.9g/l corresponded to 45% of CDW at 60h (Fig. 3). Cell dry weight was 1.97g/l at mid-stationary phase. The PHB cell content levels of 38-45% CDW were obtained during stationary phase (36-72h). Most of PHB was accumulated at 36h of incubation, but accumulation was continued during stationary phase unlike *Ralstonia* L1.

Inoculated cells were free of PHB granules at time zero for both strains (Fig. 4, inoculum, QA3 subpopulation). This population has no PHB content (FI < 0.5) and no changed SSC parameter (SSC < 4). Cell subpopulations with FI or SSC parameters above 0.5 and 4 were considered to be PHB positive and multiplication activity, respectively. PHB production did not occurred in inoculated cells, because the cells incubated in carbon free medium. Homogenous population revealed that all the cells have same properties and characteristics related to PHB production and SSC parameter. So, these cells were considered as resting cells (PHB^- and SSC^-).

Flow cytometry distinguished four subpopulations (QA1, QA2, QA3 and QA4) with different PHB content and side scatter parameter in both strains at 12h (Fig. 4, 12h, both strains). Similar to QA1, QA3 also have no PHB content but its SSC has changed (SSC > 4). SSC parameter was

similar to intracellular granule formation because fluorescence intensity of QA1 population was similar to inoculate (QA3 or resting cells). QA_2 and QA_4 subpopulations had PHB content or positive FI (FI > 0.5), but SSC parameter of QA2 was similar to A1 (SSC > 4) and A4 population. Thereby, the increasing of the QA_2 and QA_4 subpopulations were considered as indicators for PHB+, SSC+ and PHB+, SSC^-, respectively.

Therefore, QA1 did not produce PHB while had proliferating activity related to SSC parameter, QA2 had both PHB production and multiplication activity, QA3 was not active in both activities (as resting cells) and QA4 only had PHB production activity. Investigation of *Ralstonia* L1 cell subpopulations during the mid-exponential growth phase showed heterogeneous subpopulations (four subpopulations). QA2 and QA4 were dominant subpopulations (59.8% and 21.14%). QA2 had high multiplication activity and PHB content (Fig. 4, 12^{th} h). Both subpopulations contained the same PHB content.

In regard to *Sinorhizobium* AR2, two dominant population were similar to QA1 (12.72%) and QA2 (80.29%) with related multiplication activity but QA2 only could produce PHB granules. At early stationary phase of *Ralstonia* L1 major two subpopulations appeared in QA_2 and QA_4 gated regions similar to mid aid-log phase (Fig. 4, 24h). Biomass in QA_4 subpopulation were increased compared with 12^{th} h of incubation. An increase in biomass number was observed from 21.14 at 12^{th} h to 46.62% at 24^{th} h. Percentage of cells in QA_2 subpopulation decreased from 59.80% to 51.29% at 24^{th} h.

Both subpopulations were synthesizing intracellular PHB granules approximately 31.67% of CDW (Fig. 2). Both QA1 and QA3 subpopulations that were inactive in PHB synthesize, disappeared at early stationary phase of *Ralstonia* L1. Significant changes were not observed in QA2 subpopulation of *Sinorhizobium* AR2. But increase in QA4 cells number was observed from 5.92% at 12^{th} h to 20.92% at 24^{th} h. This increasing manner was simultaneous with QA1 subpopulation disappearance. Indeed, *Sinorhizobium* AR2 was at Mid-log phase (Fig. 3). Decrease of cells number in QA_2 and increase in QA_4 subpopulations continued with progress in incubation time to 72h (Fig. 4, 48 and 72^{th} h) for both strains. There were some differences between decrease and increase in percentage of QA_2 and QA_4 subpopulations. Dominant subpopulation of *Ralstonia* AR1 is QA_4 (84%). QA4 was characterized by only PHB production, not multiplication unlike QA2 subpopulation (15%) at 48-72h. Percentage of QA4 and QA2 subpopulations of *Sinorhizobium* AR2 was 72-67 and 27-31, respectively between 48-72h of incubation (stationary phase).

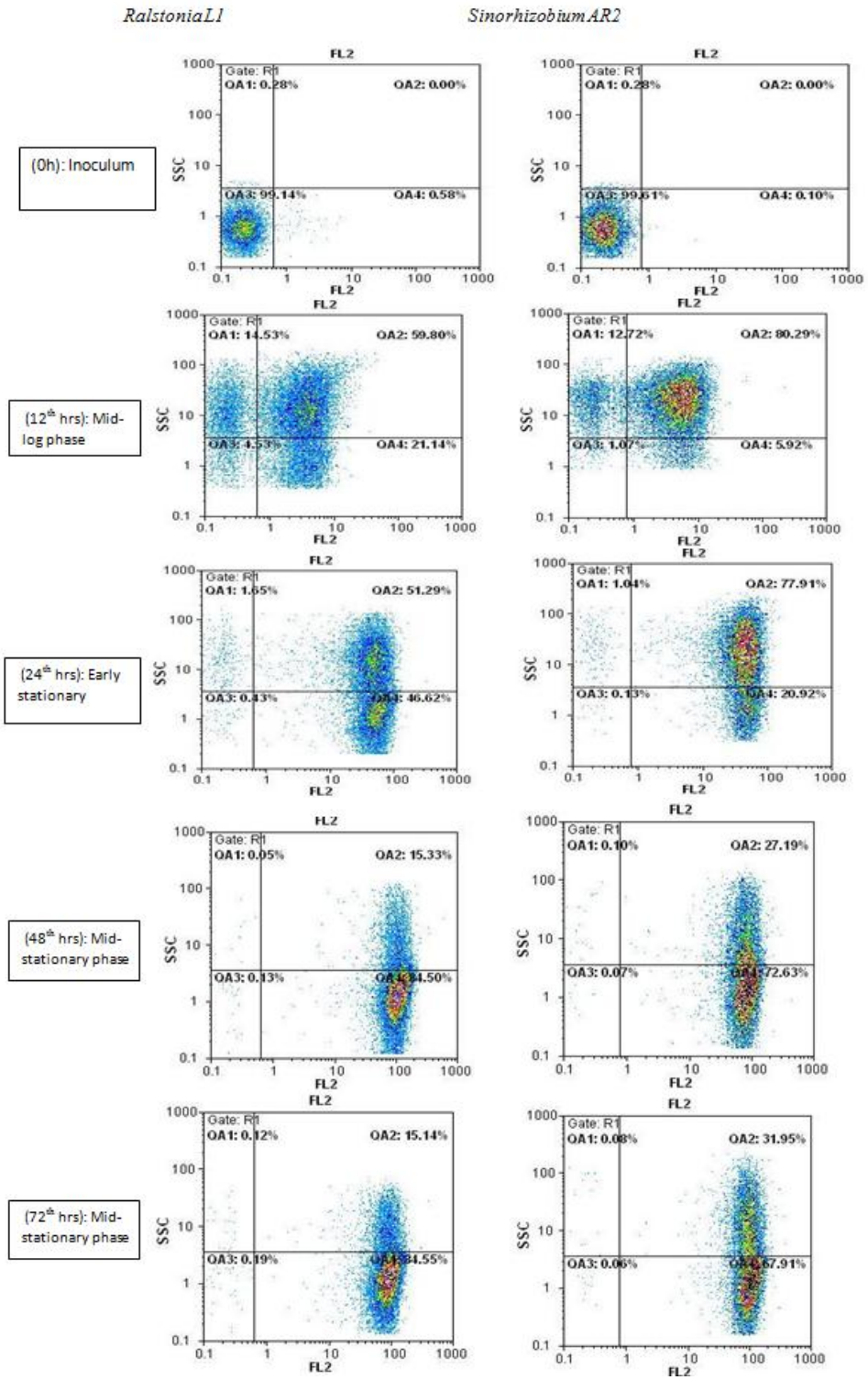

Fig. 4: Side scatter parameter Vs intracellular granules formation (FL_2 or fluorescence intensity of stained cells with Nile red) in *Ralstonia* L1 and *Sinorhizobium* AR2. Percentage of cell subpopulations is shown in top of the gates. QA_3 subpopulation is containing the cells with no PHB content and without change in SSC parameters (PHB^-, SSC^-). This subpopulation was named as resting cells. QA_2 cell subpopulation have intracellular PHB granules and changed SSC parameter (PHB^+, SSC^+) unlike QA_3 subpopulation. This subpopulation has PHB content and multiplication activity

Discussion

In the present work, a wide range of bacteria were screened extensively for PHA production. Orange fluorescence confirmed the presence of intracellular polyhydroxy-butyrate granules and provides a tool to distinguish between PHA-negative and PHA-positive bacteria by viable colony staining method [23]. Differences in the accumulation of PHAs were correlated with fluorescence intensity [26,27]. Colonies of PHA-positive strains exhibited very strong fluorescence unlike the PHA-negative strains. With the use of this test, screening of PHA producer can be fast and easy.

It is obvious that different bacterial species are known to synthesize intracellular PHB granules [28]. Genus of *Ralstonia* (specially *R. eutropha*) is the most widely employed bacterium for the production of PHAs since it can accumulate a large amount of PHAs [29-31]. Also bacteria such as *Pseudomonas*, *Azotobacter* and *Rhizobium* were isolated and used for PHB production [8]. The difference in the fluorescence intensities can be used to estimate the relative amount of intracellular PHA granule. Thereby fluorometric measurement can be used in kinetic studies of PHA granules formation [27].

PHA synthesis was initiated when carbon source is in excess or culture condition was imbalanced by nitrogen or phosphate limitations [3,4]. In this work, the PHA granule was identified as PHB by HPLC and production was initiated in *Ralstonia* L1 at early log phase when cells were dividing and multiplying. Nutrients are available and were used for cell proliferation. Rapid decline in NH_4 level is responsible for initiation of PHB production. Polymer synthesis by *Ralstonia L1*, is dependent on nutrient limitation and thereby isn't growth-associated. Flow cytometry is used to assess the change in the fluorescence intensity, forward and side scatters of cells. The change in these parameters is directly dependent on PHB formation, cell growth and multiplication [18,21-22].

The results in recent work from QA2 and QA4 population showed SSC parameter isn't related to the intracellular PHB granules. Probably the SSC parameter is related to cell division or factors contributing in DNA synthesis and active cell division. Four subpopulations with diverse characteristics were observed at mid-log phase. Each individual subpopulation has specific characteristics such as cell size, complexity and granularity related to cell biochemical and physiological states.

Heterogenic subpopulations indicate different characteristics correlated with the above factors. Whereas PHB production is intensely related to growth condition and cell physiological states, cell metabolism and physiology are important factors for polyester production [3]. Therefore different subpopulations have diverse metabolism and physiology. As a result, flow cytometry and cell arrangement techniques are suitable for organization ideal and desirable subpopulations for further biotechnological or mutational studies [22].

Conclusion

There are a lot of studies in field of PHB production but this is the first time to screen PHA producer with Nile red followed by flow cytometry for monitoring formation of granules within subpopulations.

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Conflict of interest statement: All authors declare that they have no conflict of interest.

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