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*Full Length Article*

# Corncob; the Inducer of Polyhydroxybutyrates (PHBs) of *Bacillus licheniformis*-MK656314

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## Abstract

Plastic pollution has deteriorated the ecosystem. The conventional plastics can be replaced by bacterial plastics. In this study, *Bacillus licheniformis*-MK656314 was investigated for its complete polyhydroxybutyrate (PHB) profile as well as its optimum production using agro-wastes. The vegetables and fruits garbage samples were collected to isolate PHB producing bacteria. PHB screening methods observed *Bacillus licheniformis*-MK656314 as the most efficient PHB producer. Its optimum growth conditions were: 40°C, pH 7 and glucose as a carbon source. After 24 h, its dry cell-(DCW 22.311 g/L) secreted 9.87 g/L extracellular PHB, and 3.48 g/L intracellular PHB. It showed more PHB production with corncob (55.1% extracellular and 22.5% intracellular) as compared to glucose (21.88% extracellular and 14% intracellular). SDS-PAGE showed various polypeptide bands from 34 kDa to 17 kDa when corncob was used in the medium. Optical microscopy showed the porous topology of both PHBs. Furthermore, FTIR analysis showed standard vibrations of C=O in both PHBs. DSC of both samples showed a positive heat flow during sample decomposition which represents endothermic nature of the decomposition reaction. The amount of extracellular PHB secreted by *B. licheniformis*-MK656314 was more as compared to its intracellular PHB. A slight difference in the structure of both PHBs was noticed. Corncob, a waste material, dumped in the environment can be used as a cost-effective carbon source for its production. 

**Keywords:** *Bacillus licheniformis*; Biopolymers; Bioproducts; Environmental; Soil

## Introduction

Plastics have become an important component of our daily lives (Butbunchu and Pathom-Aree 2019). They are made up of petrochemicals that are highly toxic and known to persist in the environment for longer time. Plastic pollution is increasing day by day. According to a UN Environment report (2018), nearly 200 million tonnes of plastic is being produced annually, which is approximately equivalent to the weight of entire human population currently existing on the planet earth. At the moment 9% of the plastic is recycled, 12% is incinerated while 79% of it is dumped in a landfill or natural ecosystem (Geyer *et al*. 2017). One million plastic drinking bottles are purchased every minute worldwide (Nace 2017). The WWF-Pakistan started a campaign "Beat Plastic Pollution" with a focus of creating awareness in public especially about using alternative to single-use plastic for promoting green globe (The News International 2018). Plastic in the marine environment exists in different forms as microplastic (0.05–0.5 cm), mesoplastic (0.5–5 cm), macroplastic (5–50 cm) and megaplastic (above 50 cm) which usually floats on the surface of sea until shredded down to macroplastic (Bettler *et al*. 2017). All these forms of plastic effectively pollute our biosphere by contaminating the marine ecosystem, destroying fish population, entering the food chain, clogging sewers, and becoming breeding places for mosquitoes (The News International 2018). If current trend is not controlled, it may invade our oceans more than fish by 2050 (Geyer *et al*. 2017). Many countries are making policies to ban the use of plastics. Scientists are making efforts to search suitable substitutes to replace synthetic plastics (Jain and Tiwari 2015; Munir *et al*. 2015). Traditional plastics such as polypropylene (PP) and polyethylene (PE) are in routine use due to their durability and elasticity.

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Microorganisms including bacteria are known to produce bioplastics or biodegradable plastics called as polyhydroxybutyrate (PHB). PHB belongs to polyhydroxyalkanoate (PHA), a class of biopolymer which is polyester in nature (Chaber *et al*. 2017). These biopolymers are accumulated as intracellular storage granules inside the bacteria. They have many advantages over traditional plastics as biodegradability, non-toxicity and better physical as well as mechanical properties (Bhagowati *et al*. 2015). Many microorganisms are known to produce PHB including *Halomonas* (Hertadi *et al*. 2017), *Pseudomonas*, *Bacillus* and *Alcaligenes* species (Rehman *et al*. 2015). It is reported that PHB accumulation inside cells is dependent on carbon source (Bhagowati *et al*. 2015). The efforts for commercialization of PHB had been in focus since 1950s when North-American Company W. R. Grace Co. marketed it (Barrett 2018). It has been in the market by different names as Biopol®, Nodax®, Biogreen® and Biomer® by different companies as Zeneca, Monsanto, Metabolix, Inc. (U.S.A.). Low production efficiency of microbial strains, lack of suitable purification method and high production cost (Singh *et al*. 2011; Ivanov *et al*. 2015; Munir *et al*. 2015) have limited its industrial scale production. The 50% cost of substrate for PHB production makes a difference of 12 times the cost of PP plastic (Castilho *et al*. 2009; Bhuwal *et al*. 2014). Thus, there is a high demand to search cost-effective substrate for PHB production. Organic wastes consisting of fruit and vegetable left-overs are generated all around the world on daily basis. Instead of dumping them in the environment, their suitable use should be explored (Singh *et al*. 2013). This study includes the isolation, molecular characterization of PHB producing bacterial strain from local organic-contaminated environment and optimization of its complete PHB (extracellular as well as intracellular) profile using agro-waste corncob.

## Materials and Methods

### Sample collection, isolation and purification of bacterial strains and subsequent treatments

Soil samples were collected from garbage of fruits and local vegetable market. The temperature of the samples was 37°C whereas the pH ranged from 6 to 7.5. Standard microbiological methods were used to isolate and purify bacterial colonies from them. For this purpose, Luria Bertani (LB) agar medium (peptone 10 g, yeast extract 5 g, NaCl 5 g, agar 15 g and final volume made 1000 mL with distilled water) was used. The cultural and morphological characterization of bacterial colonies obtained was performed using colony morphology, Gram staining and biochemical characterization (Cheesebrough 2001). All the experiments were performed in triplicate following completely randomized design using LB agar medium and incubation conditions of 37°C for 18–24 h.

### Qualitative screening by staining methods

All the bacterial colonies obtained (15) were screened for their PHBs production. On the basis of results, only one bacterial colony was selected which showed maximum PHB production. Further experiments were performed on the selected bacterial strain.

**Sudan black B staining:** Fresh bacterial culture was used to prepare a smear which was heat fixed on a glass slide. It was stained with Sudan black B (stain was prepared by dissolving 0.3 g Sudan black B dye in 70% ethyl alcohol), air-dried followed by washing with alcohol. Finally, 2–3 drops of safranin were placed on the smear and observed under oil immersion. Lipid granules confirming PHB granules were stained black (Collee *et al*. 1989).

**Carbol fuchsin staining:** Intracellular PHB was detected by this staining technique. Fresh bacterial culture was heat fixed on a glass slide. It was stained with carbol fuchsin stain for 45 sec. Dark color granules showed the presence of intracellular PHB (Aneja 2001).

**Nile blue A method:** It was done by the method of Spiekermann *et al*. (1999). Nutrient agar medium (per 100 mL: beef extract 1 g, peptone 0.5 g, sodium chloride 0.8 g, glucose 1 g and agar 1.5 g) plates were prepared containing 0.5 $\mu$g/mL Nile blue A reagent. The bacterial isolates were streaked on it and incubated at 37°C for 24 h. The plates were observed on a UV illuminator for orange fluorescence.

### Quantitative analysis of PHB

**Sodium dodecyl sulfate (SDS) method:** It was used for the extraction of extracellular PHB. Fresh culture was inoculated in 10 mL of LB broth and incubated at 37°C for 24 h. After 24 h, it was centrifuged at full speed for 15 min at room temperature. The supernatant was shifted to a beaker while the pellet was air-dried, weighed and stored in a refrigerator for next step. The SDS (5 g) was added to the supernatant. It was mixed well in the form of a paste, followed by incubation at 37°C for 1 h and heating at 121°C for 15 min. As it cooled down to room temperature, it was centrifuged for 15 min at full speed. The upper layer was shifted to a clean glass Petri plate and kept overnight at room temperature (Kim *et al*. 2003).

**Sodium hypochlorite and chloroform method:** This method was used for the extraction of intracellular PHB. In the pellet (saved in above mentioned step), chloroform and sodium hypochlorite were added each as 13 $\mu$L of the pellet weight and mixed gently by a micropipette. The mixture turned milky which was kept overnight at room temperature. Next day, it was centrifuged at full speed for 15 min at room temperature which resulted in three layers; the first was of aqueous Na-hypochlorite, the second of cell debris and third layer of chloroform containing PHB. The chloroform containing solution was further used by pipetting into new eppendorf. In it, a 5X mixture of 70% methanol and 30% water was added, mixed, centrifuged at

full speed for 15 min and the chloroform was allowed to evaporate at room temperature. The left-over pellet was of PHB. Sulfuric acid was added to the pellet that converted it into crotonic acid, which appeared as a brown colored precipitate (Chang *et al*. 1994; Singh and Parmar 2011).

**Optimization of selected bacterial strain**

Optimum growth conditions of the selected bacterial strain were carried out using different temperatures, pH and carbon sources.

**Optimum temperature:** For determining it, 1% fresh inoculum was added to 10 mL autoclaved LB followed by incubation at various temperatures (30ºC, 40ºC, 50ºC, 70ºC and 90ºC) for 24 h. Optical density was read at 585 nm.

**Optimum pH:** For it, the experiment was carried out at different pHs (5, 6, 7, 8 and 9). The LB broth medium was prepared in different beakers and its pH was adjusted with HCl or NaOH. The medium was dispersed into test tubes (triplicates), 10 mL each, and autoclaved. Afterwards, 1% inoculum was taken and incubated at 37ºC. The next day, OD was recorded at 585 nm.

**Optimum carbon source:** Various carbon sources used were: glucose, sucrose, maltose, lactose and fructose. In 10 mL of the LB broth, 1% inoculum and 2% carbon source were added and then the mixture was incubated at 37ºC for 24 h. Next day, OD was read at 585 nm.

**Optimization of PHB production**

Optimization of PHB production was carried out using different organic wastes (orange peels, banana peels, corncob, sugarcane left-over, potato peels and mixture of all these). Glucose was used as a control.

**Preparation of waste extracts:** First of all, the respective material was taken, washed with tap water, air-dried and cut into small pieces. They were boiled in 1000 mL distilled water till one fourth of water was left behind. This leftover solution was filtered and poured in clean sterilized glass bottle and placed in a refrigerator for further experimentation.

**Optimization experiment:** In 100 mL sterilized LB broth, 1% inoculum was added, followed by addition of 2% agro-waste extract in each of properly labeled flasks. It was incubated at 37°C for 24 h. The next day, 10 mL culture was taken from it. The SDS and chloroform methods were performed. About 1 mL of the same culture was used for protein profiling by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Laemmli 1970).

**Characterization of extracellular and intracellular PHB**

**Light microscopy:** The extracellular and intracellular PHBs were taken on a clean glass slide, stained with crystal violet, and observed under 40 X.

**Optical microscopy:** For it, extracellular and intracellular PHB samples were mixed with chloroform separately. The mixture was spread on a clean Petri dish and air-dried. It was observed using an optical microscope.

**Fourier transform infrared spectrophotometry (FTIR):** The method described by Bhuwal *et al*. (2014) was followed with slight modifications. The purified PHBs (2 mg both PHBs) were mixed with KBr separately and dried. This mixture was subjected to FTIR spectrum using a Fourier Transform IR spectrophotometer (Shimadzu, Japan).

**Thermal gravimetric analysis (TGA):** It was performed using 10 mg of PHBs in TGA instrument (SDT Q600 V8.2 Build 100) calibrated with indium. The temperature was ramped at a heating rate of 10°C/ min in air to a temperature (600°C) well above the degradation temperature of the polymer (Bhuwal *et al*. 2014).

**Differential scanning calorimetric (DSC) analysis:** For it, 10 mg of extracellular and intracellular PHB were used in standard aluminium pans. The experiments were performed using air. The samples were heated, and temperature was increased at the rate of 10°C/min (Bhuwal *et al*. 2014).

**Molecular characterization of isolated strain**

The 16S rRNA sequencing of the selected bacterial strain was got performed from Macrogen® South Korea.

**Statistical analysis**

All experiments were run in triplicate following completely randomized design. Three reading were taken, their mean and standard error of the means were calculated. The significance of the data ($P \leq 0.05$) was checked using SPSS v.17.0.

## Results

**Isolation, screening and selection of PHB producing bacterial strains**

Of 10 soil samples, 35 bacterial isolates were obtained. The staining methods revealed only six isolates positive for PHB. The bacterial isolate, named SS-1.9, was selected as the best producer of PHB out of six isolates on the basis of results obtained from the SDS and chloroform methods. The colonies showed irregular, flat elevation, creamy shiny center but transparent at borders which were irregular (rhizoidal). According to morphological, biochemical and molecular characterization, SS-1.9 was found to be *Bacillus licheniformis*. The sequence was submitted to the NCBI GenBank to obtain accession number which was registered as MK656314.

**Quantification of PHB**

The quantification of extracellular and intracellular *B. licheniformis* per dry cell weight (DCW) in g/L is given in Table 1.

**Table 1:** Quantification of total, extracellular and intracellular PHB of *B. licheniformis*-MK656314

| Features | DCW (g/L) | PHB accumulation (%) |
|---|---|---|
| Total | 22.31 | 59.83 |
| Extracellular PHB | 9.87 | 44.24 |
| Intracellular PHB | 3.48 | 15.59 |

**Table 2:** Extracellular and intracellular PHB yield per 10 mL bacterial culture using different organic wastes

| Sr. No. | Organic wastes utilized | PHB obtained (weight in grams per 10 mL culture) | |
|---|---|---|---|
| | | Extracellular | Intracellular |
| 1. | Orange peels | 3.028 | 0.123 |
| 2. | Potato peels | 1.428 | 0.065 |
| 3. | Banana peels | 2.710 | 0.171 |
| 4. | Sugar-cane leftover | 2.670 | 0.209 |
| 5. | Corncob | 7.909 | 0.524 |
| 6. | Mixture of all wastes | 1.995 | 0.188 |
| 7. | Glucose | 1.738 | 0.150 |

## Optimization of growth conditions

The optimum growth conditions of *B. licheniformis*-MK656314 were found to be 40°C, pH 7 and glucose as carbon source.

## Optimization of PHB production

Of various organic wastes used, corncob was found to be an optimum carbon source for extracellular as well as intracellular PHB production (Fig. 1a–b and 2 and Table 2).

## Protein studies

Different organic wastes caused the expression of different proteins by *B. licheniformis*. Their expression patterns as observed using SDS-PAGE are shown in Fig. 3.

## Characterization of extracellular and intracellular PHB

### Light microscopy

The extracellular and intracellular PHB appeared as black structures in light microscopy (Fig. 4a–b).

### Optical microscopy

The surface of extracellular and intracellular PHBs appeared porous (Fig. 4c–d).

### FTIR

The spectrums of both extracellular and intracellular PHB are shown in Fig. 5. For characterization of PHB, the FTIR spectrum of extracellular PHB showed the presence of peaks at 3446.08 $cm^{-1}$, 2916.58 $cm^{-1}$, 1656.29 $cm^{-1}$, 1467.63 $cm^{-1}$ and 1216.85 $cm^{-1}$ which correspond to hydroxyl (-OH) stretching, aliphatic (C-H) stretching, carboxylic (C=O)

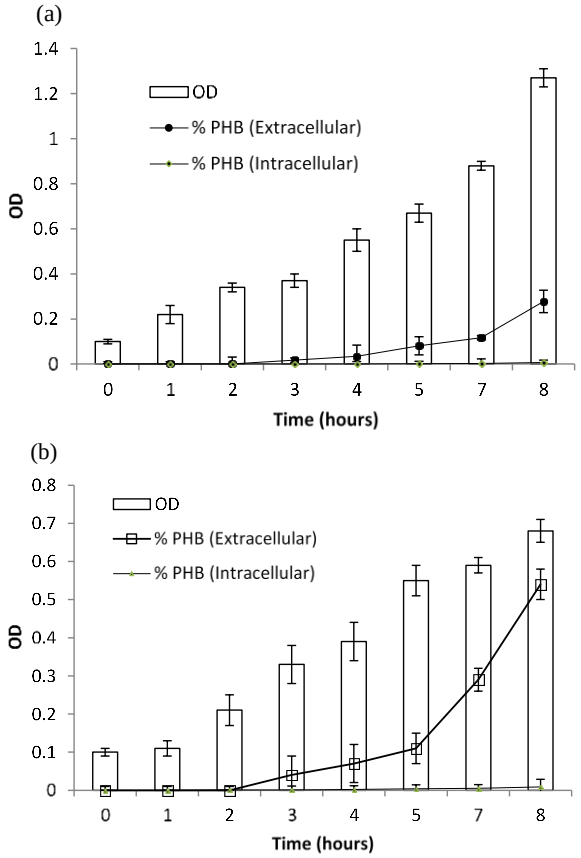


**Fig. 1:** Optical density showing the growth of cells at 587 nm and extracellular and intracellular PHB production (%) in the presence of (**a**) glucose, and (**b**) corncob as carbon sources

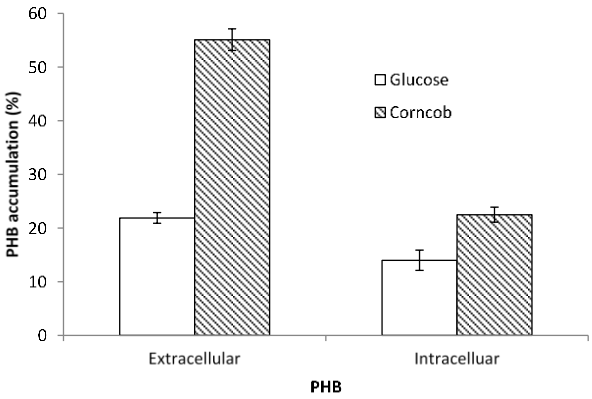


**Fig. 2:** Comparison of extracellular and intracellular PHB accumulation (%) in the presence of glucose and corncob

stretching, methyl ($CH_3$) stretching and again aliphatic (C-H) stretching, respectively. The FTIR analysis of *B. licheniformis* intracellular PHB showed the peaks at 3364.97 $cm^{-1}$, 2923.92 $cm^{-1}$, 1633.77 $cm^{-1}$ and 1077.81 $cm^{-1}$ corresponding to hydroxyl (–OH) stretching, ester (CHO) stretching, carboxylic (C=O) stretching and again ester (CHO) stretching, respectively.

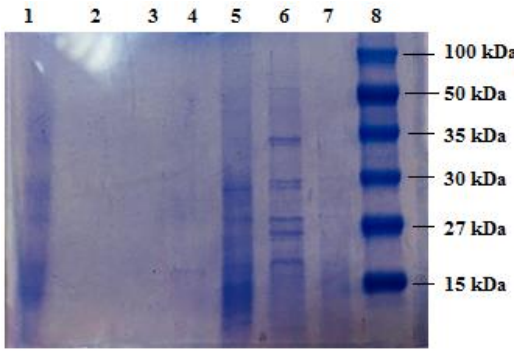


**Fig. 3:** Protein bands of *B. licheniformis*-MK656314 when grown in the presence of different organic wastes. The labeling of the wells is as follows: 1 = orange peel, 2 = potato peel, 3 = banana peel, 4 = mixture of all wastes, 5 = glucose, 6 = corncob, 7 = sugarcane and 8 = protein ladder

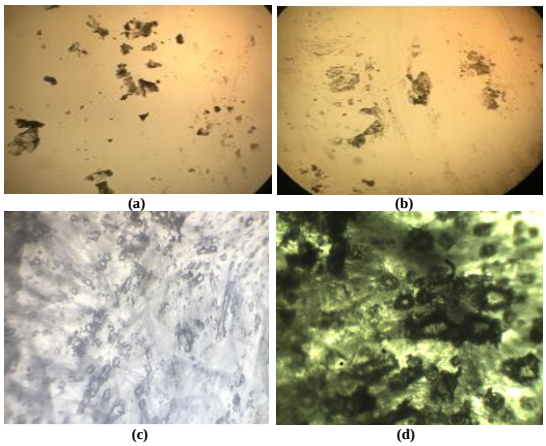


**Fig. 4:** Light microscopy of (**a**) extracellular, and (**b**) intracellular PHB. Optical microscopy of (**c**) extracellular and (**d**) intracellular PHB

## TGA/DSC

The TGA of the sample was performed to check its thermal stability at heating rate of 10°C per min in the temperature range of 0–600°C. The results (Fig. 6) showed that the decomposition of the sample started at 25°C and continued till 200°C. During this initial decomposition process, weight loss occurred in extracellular and intracellular PHB, which may have been due to loss of water and gaseous components from the sample. Extracellular PHB shows little stability up to 214.64°C and then weight loss occurred. At 299.24°C, the amount of the sample remained was 37.33%. As far as intracellular PHB was concerned, it showed little stability up to 171.07°C. Finally, at 290.16°C, the sample amount remained 58.12% by weight (Fig. 6). Similarly, DSC of both samples showed a positive heat flow during sample decomposition, which represents endothermic nature of the decomposition reaction. Tg, Tc and Tm of both PHBs are given in Table 3.

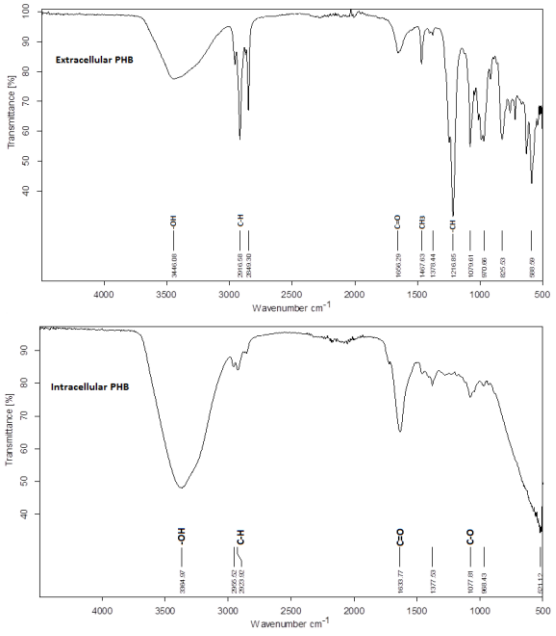


**Fig. 5:** FTIR spectra of extracellular and intracellular PHB of *B. licheniformis*-MK656314

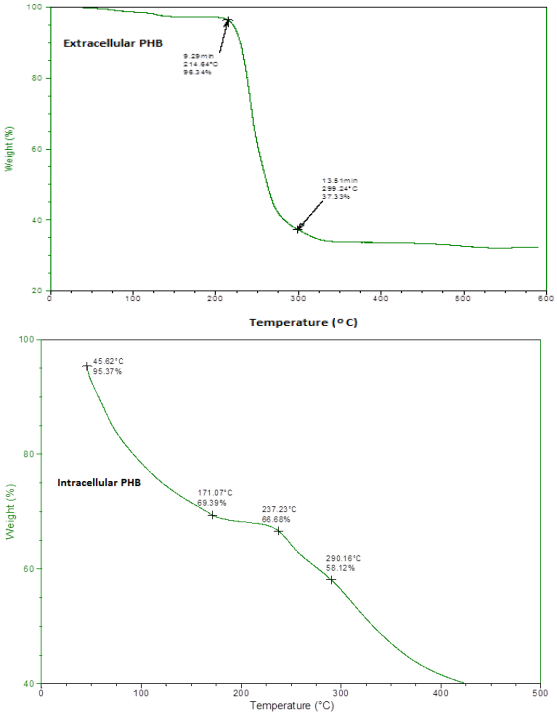


**Fig. 6:** DSC-TGA thermograms of extracellular and intracellular PHB of *B. licheniformis*- MK656314

**Table 3:** Differential scanning calorimetric (DSC) properties of extracellular and intracellular PHBs

| Properties | Tg (°C) | Tc (°C) | Tm (°C) |
|---|---|---|---|
| Extracellular PHB | 102.2 | 194.72 | 355.08 |
| Intracellular PHB | 170.0 | 80.0 | 207.1 |

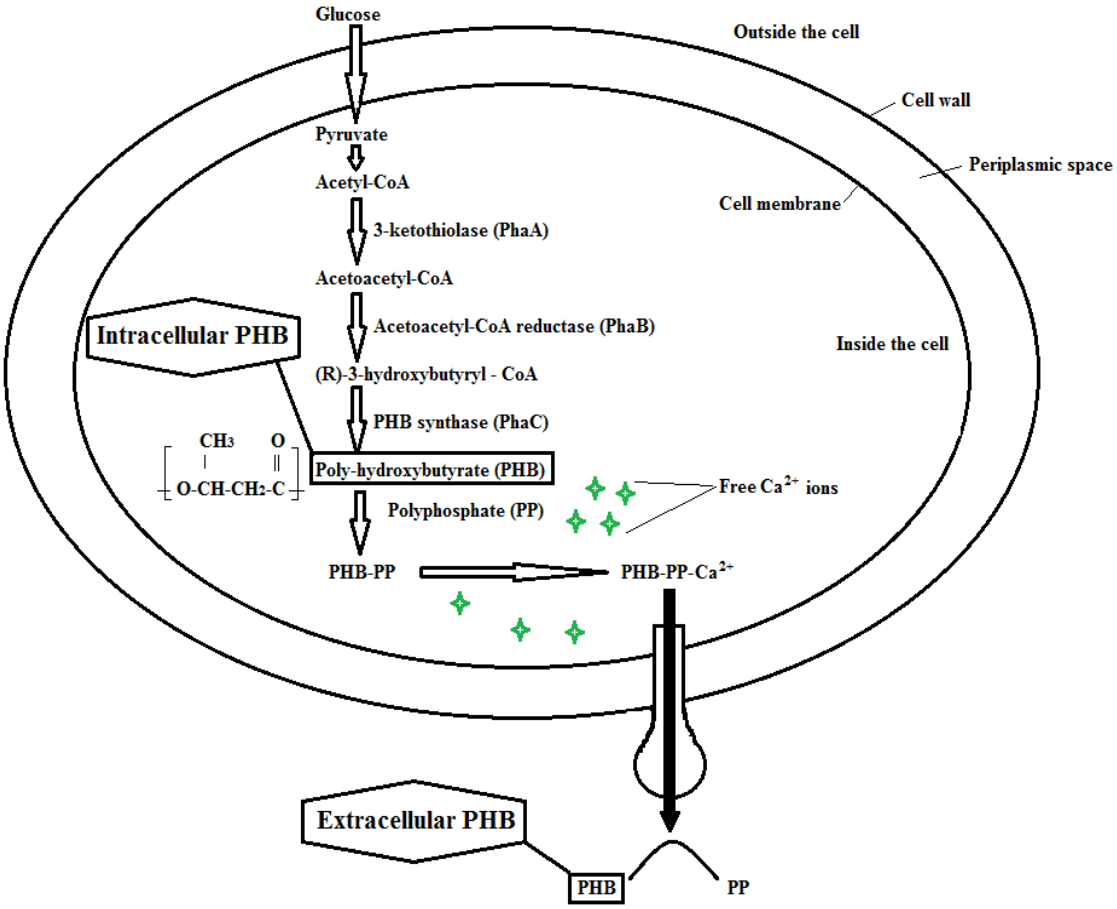


**Fig. 7:** Hypothetical model of secretion of extracellular PHB outside cytosol after being synthesized intracellularly

## Discussion

Bacterial plastics as an alternative to petroleum-based plastics are studied by different researchers (Arikan and Bilgen 2019). PHB producing *B. licheniformis* was successfully isolated from local environment. The 22.311 g/L *B. licheniformis* cells (DCW) were found to secrete 9.87 g/L extracellular and 3.48 g/L intracellular PHB. As PHB is an intracellular lipid granule, the question arises as to whether it is intracellular, then how it is released outside the cytosol. It can be explained by considering the role of calcium ($Ca^{2+}$) ions that are known to play a myriad of significant physiological roles in bacteria like host pathogen interactions, virulence, chemotaxis, cell differentiation and membrane transport (Dominguez 2018). The hypothetical model of secretion of extracellular PHB outside the cytosol is shown in Fig. 7. PHB binds to polyphosphates (PP) present in the cytosol and forms a PHB-PP complex (Ripoll *et al.* 2004). This structure then binds to $Ca^{2+}$ ions and is exported out via ATPase channels. Intracellular PHBs exist in amorphous "rubbery" form, whereas extracellular PHBs are in amorphous crystalline form (Handrick *et al.* 2004). Both PHBs production was enhanced by using corncob as a carbon source in the medium (Table 2). The production of PHB can be related with the corncob as a carbon source because it contains xylose and arabinose (Pointner *et al.* 2014). *B. licheniformis* prefers xylose and arabinose (Mota *et al.* 2002) over glucose (Scheler and Hillen 1994) for PHB production. Our study is in partial agreement with Rehman *et al.* (2016) who reported glucose and fructose as preferred carbon sources for *B. cereus* NRRL-B-3711 for PHB production. The positive effect of xylose on PHB production was observed by Singh *et al.* (2011). Interestingly, by using corncob, protein bands of 34 kDa, 29.5 kDa, 29.3 kDa, 28 kDa, 27 kDa and 17 kDa were obtained. It showed more protein expression as compared to that of glucose where only two bands of 29 kDa and 27 kDa were obtained. A detailed study of these protein bands can help us to establish their positive correlation with PHB production. At the moment, appropriate literature does not exist which can support our findings. Both extracellular and intracellular PHBs were same, *i.e.*, black membranous structure when observed under the light microscope (Fig. 4 a–b). The optical microscopy revealed porous topology of

both PHBs (Fig. 4 c–d). Râpă *et al.* (2011) also reported porous PHB. In contrast, Rehman *et al.* (2016) found a smooth film without any crack which is indicative of brittleness of PHB material. Porous PHB is in limelight with respect to bone tissue engineering for addressing small bone defect replacement (Tan *et al.* 2016; Senatov *et al.* 2017; Petrovova *et al.* 2019). The confirmation of PHB was performed by FTIR spectra of extracellular and intracellular PHB. In this study, FTIR spectra were recorded in the range of 4000–500 $cm^{-1}$. The presence of C=O group was the confirmation of PHB (Bhuwal *et al.* 2014; Bhagowati *et al.* 2015; Rehman *et al.* 2016; Hertadi *et al.* 2017) which was in agreement with our study (Fig. 5). According to TGA/ DSC results, the endothermic nature of both types of PHBs showed that it could absorb heat (Fig. 6 and Table 3). Higher Tm revealed that microcrystals of PHB molecules pack more tightly in perfect structures. Tm obtained here is more than that (109.4°C) reported by Bhagowati *et al.* (2015) and (120°C) Singh *et al.* (2013). Higher Tm showed the thermal characterization of PHB as thermostable biopolymer (Rehman *et al.* 2016).

## Conclusion

*B. licheniformis*-MK656314 was found to produce PHBs whose production can be enhanced by using corncob which is agro-waste material. Further characterization of PHBs by NMR and XRD can help us in establishing its potential applications in industry.

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