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Screening of Poly-β-hydroxybutyrate (PHB) Producing Bacteria and yeasts from locally vendored 'Whey' – a biowaste sample

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ABSTRACT

Whey- a byproduct of cheese manufacture, has several commercial uses. The present study explored the microbial load of the whey and the possible existence of biopolymer (PHB) producing microorganisms in this byproduct. Whey samples collected from different vendors in the locality of Mirpur, Dhaka, were brought into the laboratory, and the microbial load of each sample was determined by serial dilution technique using nutrient agar and YEPD medium. From 30 colonies, 32 strains were preliminarily isolated. Out of 32 isolates, fifteen exhibited pink fluorescence on mineral medium, indicating the presence of PHB production inside cells. Among these, seven strains were identified as members of the genera *Candida tropicalis;* based on molecular characterization. The remaining 8 isolates were bacteria. Yeast isolates were obtained from locally vendored whey samples, whereas bacterial strains were predominant in commercial samples. This work can be considered the first step in developing an eco-friendly bio-refinery pathway for the cheese industry.

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Conflict of interest: The authors declare that they have no conflicts of interest.

Introduction

Plastic production and accumulation have devastating environmental effects, and consequently, the world needs environmentally friendly plastic substitutes[1]. Up to 23 million metric tons of non-degradable plastics are being accumulated in the environment per year,. It is predicted that the amount could be doubled by 2025 as it is approaching an irreversible 'tipping point'[2].

Among biodegradable plastics, Polyhydroxyalkanoates (PHAs) are a group of biodegradable polymers of biological origin[3]. PHAs are accumulated as a carbon or energy storage material in various microorganisms usually under the condition of limiting nutritional elements such as N, P, S, O, or Mg in the presence of excess carbon source[4]. The main advantage of PHAs is that, it is completely degraded to water, carbon dioxide and methane by anaerobic microorganisms in various environments such as soil, sea, lake water and sewage and hence are easily disposable without harm to the environment[5].

At the beginning of this new millennium, the development of biodegradable packaging materials from waste or by-products, particularly in EU countries has received increasing attention[6]. For instance, LIFE WHEYPACK European project aims to produce Poly-β-hydroxybutyrate (PHB), which is the simplest form of PHAs; from the whey surplus for its application as biodegradable packaging material in the cheese industry (Bioplastics MAGAZINE, 2016-10-07)[7]. Bioplastics Market Data[8] shows that biodegradable plastics, such as PLA, PHA, and starch blends, are projected to grow 1.8 million tonnes in 2025 from around 1.2 million tonnes in 2020.

Whey constitutes the main by-product of the dairy industry and it is mainly composed of lactose (39–60 g/L), fats (0.99–10.58 g/L), proteins (27–60 g/L), and mineral salts (4.6–8 g/L)[9,10]. It is obtained by precipitation and removal of milk casein during the cheese-making processes. According to Nikodinovic-Runic et al.[11], approximately 120 million tons of whey are being produced annually worldwide, of which only 50% are used as human and animal feed. The remaining whey needs to be disposed of, which raises environmental concerns due to its relatively high organic load.

In this sense, the high organic content represents a great potential to become an important resource to explore. The value of using surplus whey or whey lactose for PHA production is clear as it would dramatically decrease the costs of PHA production, without competing with the production of food for humans and simultaneously solving an environmental problem. In the last decade, Berwig et al.[12] demonstrated that Alcaligenes latus can convert whey lactose into PHA with a productivity1 g L−1 h−1. Obruca et al.[13] tested the direct conversion of cheap waste cheese whey into PHB by Bacillus megaterium CCM 2037. Thammasittirong et al.[14] on the other hand, used sugarcane juice for economical PHB production by B. thuringiensis B417-5. However, the efficient use of whey for PHA production is still hindered by issues such as whey pre-treatments and choice of PHA producing strain[1].

Locally vendored whey contains many indigenous bacteria and yeasts which may have the potential to become suitable PHA-producing strains. Moreover, PHB synthesis in eukaryotic cells is not well documented compared to bacteria. Therefore, using whey as a raw material to produce bioplastics like PHB can be considered an important area of investigation. With this aim, this study was undertaken to isolate and screen polyhydroxybutyrate (PHB, a biodegradable plastic) producing microorganisms from commercial and locally available whey which is a popular drink in Bangladesh. The microbiological status of biowaste ‘whey’ available in the market was also determined. The study also focused on screening and molecular identification of PHB producing bacteria and yeasts from the sources based on fermentation performance.

Method

Sample collection

Whey samples in this study were collected from three different areas in Dhaka city viz. Mirpur 1, Kazipara and Mirpur 14 from local vendors. Samples were kept in pre-sterilized flasks and beakers and were brought to laboratory immediately for analysis. Commercial whey samples were purchased from local supermarket (Fig. 1).

Bacterial and Yeast strains were isolated by spread-plating on Nutrient agar (Difco) and Yeast Extract Peptone Dextrose (YEPD) medium (g/L) containing Dextrose 20g Peptone 20g Yeast extract 20g Agar 20g. Nutrient Agar (NA, Difco) and Yeast Extract Peptone Dextrose (YEPD) media were prepared in flask and pH was adjusted to 5.5 by digital pH meter (JENWAY, 3510). Collected samples were serially diluted and plated on nutrient agar for enumeration. Colonies that developed on agar were counted by a Stuart SC6PLUS colony counter. All isolates were purified, named temporarily and maintained in nutrient agar slants

for further study. For preliminary microscopic investigations, these were stained by two different methods-simple staining (using safranin) and differential staining (gram staining).

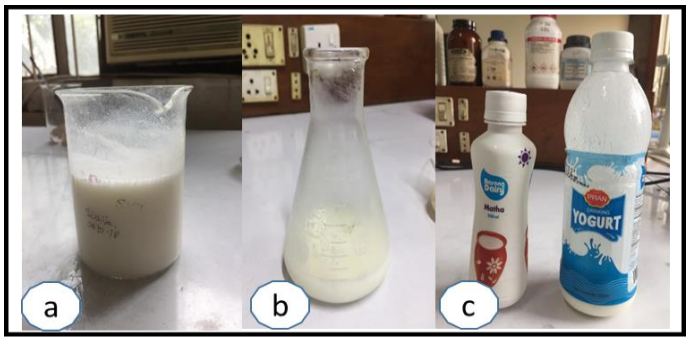

Fig 1. Local whey samples (a, b) and commercial whey samples(c) used in this study

Rapid Screening of Isolates for PHB Production by Plate Assay Method

All isolates were qualitatively screened for PHB production following the viable colony method of screening in mineral salt medium[15] using Nile red. Nile red is the fluorescent oxazone form of Nile blue A. The sterilized mineral salt medium was supplemented with 0.5 mg Nile red stain (dissolved in 1ml Dimethysulfoxide) to give a final concentration of 0.1 ml dye per ml medium according to Spiekermann et al.[16]. After inoculation, petri dishes were incubated for 2-3 days at 37˚C. The plates were exposed to ultraviolet light (360 nm) using an UV photometer. Visual observation of Pink fluorescence in the growth confirmed the accumulation of PHB.

PHB production in shake flask using 1% glucose

PHB production was evaluated in a simple discontinuous, batch culture process using simple sugars e.g., glucose as carbon source. PHB biosynthesis was performed in a 100-mL flask pre-sterilized with 20 ml mineral medium. 1% glucose solution was filter sterilized and was added before inoculation with 10% (v/v) of overnight culture and flasks were incubated at 37˚C, 100 rpm/min for 48 h. Samples (2 ml) were taken at 0, 24 and 48 hours. It was then centrifuged at 4000 rpm for 15 minutes. The culture supernatant was used for determination of reducing sugar whereas pellet was used for measuring dry cell mass and concentration of PHA. All analyses were carried out in duplicates and average values are given in Table Nos. 1-3.

Determination of Reducing Sugar of the Culture Broth

Reducing sugar in the fermented and non-fermented substrates at their various stages of fermentation were determined by the DNS method[17]. From each 0, 24 and 48 hour samples, 1ml of appropriately centrifuged and diluted culture supernatants were mixed with 3ml of DNS reagent. The mixture was then heated in boiling water bath for 15 minutes to develop the red-brown color. Then optical density was taken for different concentration of glucose at 540 nm against reagent blank. A standard curve using 0.0-0.5 g/100 ml glucose was prepared and this was used to determine the glucose concentration during fermentation experiment.

Quantitative estimation of PHA

Determination of PHB accumulation in bacteria and yeast was based on the degradation of PHB with sulfuric acid to crotonic acid, and measurement of the reaction at 235 nm by spectroscopy[18]. The dried, pre-weighted bacterial and yeast cells were dissolved in 1 ml H_2SO_4 and heated for 1 hour at 90 ºC in 1.5 ml Eppendorf tubes. The H_2SO_4 hydrolysed and dehydrates the PHA to form crotonic acid. The red brown colour mixture (Fig 2) was then cooled to room temperature and filtered with a syringe filter to remove the cell debris. The

filtered sample was diluted to detect the absorbance at 235 nm[19] with the UV visible spectrophotometer (Schimadzu CPS-240a). A standard curve was prepared with pure PHB and PHB concentration of the samples was determined by using this curve[18]. From this absorbance, PHB concentration and % PHB yield in every sample was measured.

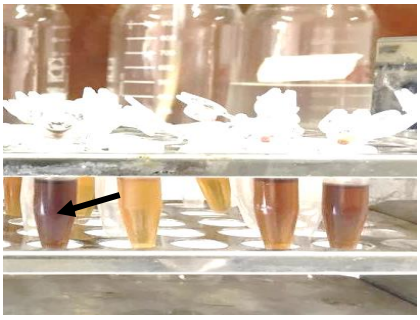

Fig 2. Sulfuric acid digestion of dry cell for PHB determination. Arrow indicates acid digested sample

Biochemical characteristics of the selected isolates

For bacterial isolates, biochemical tests *viz*. Catalase, Oxidase, Indole Production, Methyl Red and Voges Proskauer tests were carried out as per the method given by Cappuccino and Sherman[20] with 24-48h old cultures. Identification of yeast species was accomplished by BLAST-querying the GenBank database at http://www.ncbi.nlm.nih.gov/blast which generally achieved similarity scores usually between 92% and 99%.

Results and Discussion

pH and microbial count of Whey Samples

Whey samples collected in this study were found to be acidic in nature. The values of pH of local and commercially available whey samples were found to be in the range of 3.5-5.0. The pH of locally available whey samples nos. 1, 2 and 3 were 4.94 ± 0.1, 4.00 ± 0.2 and 3.55 ± 0.2, respectively. On the other hand, the pH of commercial whey samples A and P were found to be 3.73±0.25 and 3.55±0.11, respectively. Local whey sample Nos. 1, 2 and 3 showed microbial count of 10×10^6, 54×10^5, 9×10^6 cfu/ml, respectively, which are slightly lower than commercial whey A and P samples with values of 9×10^6 and 27×10^7 cfu/ml, respectively.

Screening of PHA producing isolates from Whey samples

All isolates were qualitatively tested for PHB production. As shown in Table 1, out of 32 isolates, only 15 isolates showed pink fluorescence indicating the presence of PHA. Isolate nos. AR1, AR2, AI1, AI2, PI1, PI2, PI3, PI, L4, L7, L12, L16, L19, L22 and L23 were found to give pink fluorescence under UV (360 nm) with glucose as carbon sources as shown in Table 1 and Fig 3.

Table 1. Fluorescent intensity of PHA producing isolates after screening with Nile red

Category	Isolate Nos.	Fluorescence intensity for glucose carbon sources*
Local whey sample	L4, L19, L22	+
	L7, L16, L23	++
Commercial whey sample	AR1, AR2, AI1, AI2, PI3, PI4	+
	PI1, PI2	++

* + poor, ++ moderate and +++ good

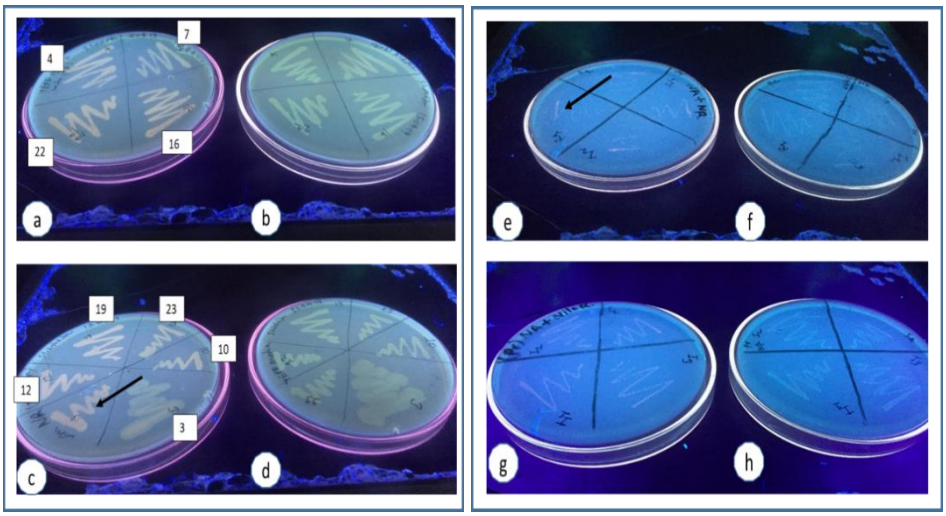

Fig 3. Photographs showing isolates grown on mineral salt media agar plates without Nile red (b, d, f, h) and with Nile red (a, c, e, g) under UV exposure at 360 nm. Pink fluorescence (arrow) indicated presence of PHA.

Microscopic observation, gram reaction and colony morphology of isolates from whey samples

Microscopic observation confirmed the presence of 8 bacterial isolates and 7 yeast isolates from whey samples after screening. Most of bacterial isolates were rod shaped and two isolates were (AR1, AR2) were coccoid. All isolates were Gram positive. In literature, gram-positive bacteria are better PHA sources for the medical field, since they lack outer membrane lipopolysaccharide (LPS), which induces strong immunogenic reactions[21]. Cells of AR1 were slightly curved and had tendency of forming coils as shown in Fig 4. AR2 is coccoid, AI1 and AI2 are thin rod chain, PI1 is thin rod like and the rest PI2, PI3, PI4 are rod shaped. solated *Lactococcus*, *Klebsiella*, *Pseudomonas*, *Enterobacte*r and *Enterococcus* genera from whey samples[22]. Oksuz et al.[23] studied the effect of SCO7613 gene region in *Streptomyces coelicolor* for PHB accumulation.

Cell morphology of all yeast isolates were similar in size and shape. Cells of L16 and L22 isolates stained with crystal violet and safranin, respectively, are shown in Fig 4. Accumulation of PHB granules in yeast isolates were seen under 100x immersion oil objective lens in light microscope. Microscopic observation of all isolates revealed cell shape and arrangement.

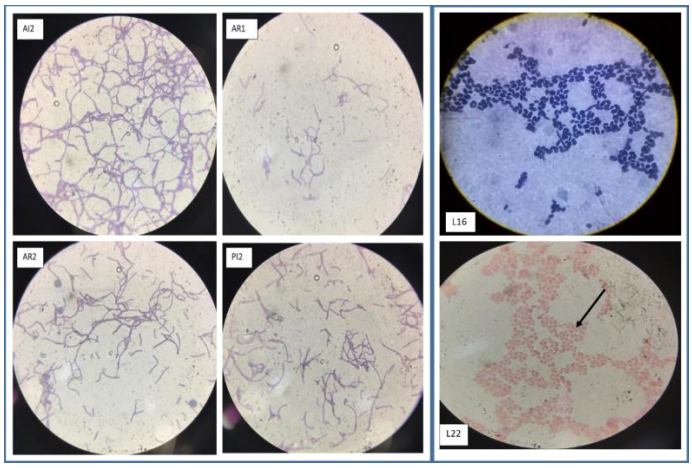

Fig 4. Photographs showing of gram positive bacterial isolates Nos. AI2, AR2, AR5, PI2 and cell morphology of yeast isolates Nos. L16 and L22 from whey samples under microscope

PHB production of bacterial isolates in shake flask using 1% glucose

In this study, PHB production was evaluated by growing bacterial isolates in mineral media containing 1% glucose as the only carbon source. The growth and PHB production in flasks after incubation will imply that the isolates can utilize glucose as energy as well as for storing excess carbon inside the cells into PHB granules. Bacterial growth in terms of optical density (OD) was determined for 0, 24 and 48 h using spectrophotometer at 600 nm. In general, bacterial growth increased with the incubation period. At 48 h incubation period, PI1 showed maximum visible growth (OD= 0.78) whereas PI3 showed the least OD value 0.44 which means minimal growth. PI2 and PI4 isolates showed moderate growth (OD = 0.73, 0.54, respectively). In commercial whey A samples, at 48 h incubation period, AR2 showed maximum visible growth (OD= 0.61 nm) whereas AI2 showed the least OD value of 0.22. AR1 and AI1 isolates had moderate growth (OD = 0.58, 0.30, respectively). During fermentation, growth of bacteria increased and the concentration of glucose was found to decrease gradually. In commercial whey P sample, PI1 isolate consumed the highest amount of glucose and after 48 h, glucose concentration was 5.14 g/L. In the commercial whey A sample, AI2 isolate consumed the highest amount of glucose than other isolates. Among 8 bacterial isolates, AR2 showed the promising production of PHA with the content of more than 41% as given in Table 2.

Table 2. Changes of cell dry weight and PHA content over time in Bacterial isolates

Code of isolates	Incubation period (h)									
	24					48				
	OD value	CDW (mg/L)	Glucose conc. (g/L)	PHA Conc. (mg/L)	% PHB yield	OD value	CDW (mg/L)	Glucose conc. (g/L)	PHA Conc. (mg/L)	% PHB yield
AR1	0.45	4.5	6.0	0.206	5	0.58	2.3	1.22	0.303	11
AR2	0.73	3.7	6.77	0.017	1	0.61	1.9	1.78	0.778	41
AI1	0.15	2.6	5.32	0.203	8	0.30	1.5	2.74	0.467	31
AI2	0.17	1.8	6.19	0.146	8	0.22	1.9	0.55	0.186	10
PI1	0.44	1.1	7.78	0.111	10	0.78	1.9	5.139	0.139	7
PI2	0.59	1.7	8.99	0.298	17	0.73	2.3	6.63	0.187	8
PI3	0.28	4.3	7.33	0.118	3	0.44	1.4	6.44	0.303	22
PI4	0.36	2.8	8.61	0.143	5	0.54	1.4	7.04	0.292	21

PHB production of yeast isolates in shake flask using 1% glucose

In case of yeast isolates, PHB production was evaluated by growing in mineral media containing 1% glucose as the only carbon source. The growth and PHB production after incubation will imply that the isolates can utilize glucose as energy source and also for storing excess carbon inside their cells as PHB granules. At 48 h incubation period, L7 showed maximum OD of 4.84 whereas L23 showed the least OD value of 3.65 which means minimal growth. The rest of the isolates had intermediate growth rate. Dry cell mass and concentration of PHA were determined for all isolates during batch fermentation with 1% glucose. Cell dry weight varied over time and the capacity of PHA production was also variable for each isolates. At 24 hour incubation period, L12 showed highest PHA production with 36% accumulation as shown in Table 3. In local whey sample, yeast isolates consumed more glucose than bacterial isoaltes. Among 7 isolates, almost all glucose was consumed by L12 isolate after 48 hour.

Table 3. Changes of cell dry weight and PHA content in yeast isolates during fermentation

Yeast Isolate	Incubation period (h)									
	24					48				
	OD value	Glucose conc. (g/L)	CDW (mg/L)	PHA Conc. (mg/L)	% PHB yield	OD value	Glucose conc. (g/L)	CDW (mg/L)	PHA Conc. (mg/L)	% PHB yield
L4	2.6	4.67	1.9	0.546	28	3.90	2.66	3.9	0.280	7
L7	3.13	0.36	3.1	0.087	2	4.84	0.7	8.2	0.078	1
L12	2.19	5.78	2.2	0.804	36	3.92	0.25	3.2	0.430	13
L16	2.42	4.98	2.8	0.139	4	4.32	0.57	3.6	0.100	3
L19	3.13	2.62	2.1	0.253	12	4.00	0.19	3.2	0.230	7
L22	2.43	6.99	2.2	0.100	5	4.37	1.47	2.6	0.179	7
L23	1.55	5.88	1.6	0.176	11	3.65	3.12	4.1	0.185	5

Biochemical characteristics and molecular identification of isolates

Biochemical characteristics e.g., oxidase, catalase, Indole, Methyl Red and Voges Proskauer were performed for bacterial isolates in this study. All isolates were Catalase, Oxidase and VP negative and exhibited cherry red ring on surface after addition of reagent which indicated positive result for indole test. All isolates were MR positive. Based on molecular characterization, seven yeast isolates were identified as strains of the genera Candida tropicalis.

Conclusion

The possibility of using isolated bacteria and/or yeast strains to produce environmentally sustainable bioplastic could also solve the disposal of biowaste like whey. In this study, it was found that whey contains many yeasts and bacteria which were capable of producing PHAs. 15 out of 32 isolates exhibited pink fluorescence on mineral medium, indicative of PHA production. Out of 15 isolates, 8 strains were bacteria, rest were found to be yeasts cells. In terms of PHA content, L12 isolates showed highest accumulation (36%) among 7 yeast isolates after 24 hr using 1% Glucose as substrate. Safak et al. [24] isolated 16 yeast strains from Komubucha tea in Turkey. They found that the PHA accumulation in these isolates were in the range of 0.5 to 16.67%. In our study, among bacterial isolates, highest PHA content was 41% for AR2 after 48h. Kahraman et al.[25] discovered PHB-producing extreme halophilic archaeon Haloferax sp MA10 at İzmir's Çamaltı Saltern for the first time and showed that acetate has a positive effect on PHB yield. However, a more in-depth study is required to know the most suitable carbon sources and nitrogen sources of these isolates for maximum PHB production. Further study to produce PHAs using whey as substrate is necessary to make these strains economically viable.

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Author contributions

All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by Shishita Zahan Zisha. The first draft of the manuscript was written by firth author too. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.