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Production and characterization of medium-chain-length polyhydroxyalkanoate copolymer from Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620

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ABSTRACT

Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620 was found to produce a distinctive medium-chain-length polyhydroxyalkanoate (MCL-PHA) copolymer when grown on structurally unrelated carbon sources including glycerol. The maximum MCL-PHA copolymer yield was obtained about 52.18 ± 4.12% from 7.95 ± 0.66 g/L of biomass at 144 h of fermentation when 3% glycerol was used as sole carbon and energy source during the laboratory-scale bioreactor process. Characterization of the copolymer was carried out using fourier transform infrared spectroscopy (FTIR), gas chromatography–mass spectrometry (GC–MS), proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance spectroscopy (NMR), gel permeation chromatography (GPC), differential scanning calorimeter (DSC) and thermo-gravimetric analysis (TGA). The copolymer produced by *Pseudomonas* sp. PAMC 28620 consisting of four PHA monomers and identified as 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) and 3-hydroxytetradecanoate (3HTD). An average molecular weight of the copolymer was found approximately 30.244 kDa with polydispersity index (PDI) value of 2.05. Thermal analysis showed the produced MCL-PHA copolymer to be low-crystalline (43.73%) polymer with great thermal stability, having the thermal decomposition temperature of 230 °C–280 °C, endothermic melting temperature (T_m) of 172.84 °C, glass transition (T_g) temperature of 3.99 °C, and apparent melting enthalpy fusion (ΔH_m) about 63.85 J g^{-1}.

1. Introduction

Polyhydroxyalkanoates (PHAs) are group of biopolymer produced by numerous kinds of bacteria as an energy and carbon reserve materials in response to nutritional limitation, stressful conditions, and/or presence of excess carbon source in the growth medium [1,2]. PHAs are retaining numerous industrial and bulk applications due to their ecofriendly nature and PHAs also gained a worldwide attention since they are completely biodegradable, biocompatible, and it can be produced from renewable resources [1,3]. PHAs are composed of repeating monomeric units of hydroxyl fatty acids (HFA) as linearized structures. Generally, the monomeric structure and arrangement of PHA is predisposed by numerous factors, mainly PHA synthases, carbon source, and the PHA metabolic pathways [4]. Mostly, PHAs are classified into two major classes such as short-chain length (SCL) and medium-chain length (MCL) polymer according to the number of carbon atoms present in the HFA monomers. The SCL-PHA and MCL-PHA polymers consist of 3–5 and 6–14 carbon lengths, respectively [2]. MCL-PHAs and their copolymers are structurally, thermally, and mechanically diverse than SCL-PHA, however, researches on MCL-PHA, still remain limited, mostly due to the lack of efficient MCL-PHAs producing bacteria and expensive fermentation strategies for commercial production [5,6].

Traditionally, fatty acids have been used as the main substrate (structurally related) for the bacterial synthesis of MCL-PHAs [7]. However, the production of MCL-PHAs in large scale is limited due to the high cost of substrates (fatty acids). Several low-

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cost (structurally unrelated) substrates are being investigated to optimize the industrial production of MCL-PHAs from wild-type and metabolically engineered bacterial systems [8–10]. Glycerol, a large co-product stream from biodiesel production, is one of the most fascinating substrate for MCL-PHAs production due to huge rise in biodiesel production worldwide and also economically cheaper than fatty acid-based substrates [11]. Glycerol has been tested as fermentation feedstock for the production of poly-3-hydroxybutyrate (PHB) from numerous bacterial systems [12]; however, the large-scale production of MCL-PHAs using glycerol as main substrate is relatively less focused [11,13]. At present, efficient bacterial systems that utilize glycerol as a main substrate to produce MCL-PHAs with better thermal and physical properties are required to develop MCL-PHAs biomaterials for commercial and biomedical applications.

Microorganisms that exist in cold environments are well adapted to various fluctuating stressful conditions. For example bacteria living in polar environments have to tolerate extreme conditions such as low temperature, low nutrient availability, and high UV radiation [14]. It has been strongly believed that the PHA synthesis is one of the survival mechanisms of the bacteria to live in stressful and unstable environments [15,16]. Moreover, the metabolic and catabolic PHA pathways possibly might increase the bacterial fitness in fluctuating environments [17]. In recent decades, the production of PHA from different kinds of wild type bacteria and their PHA biosynthesis metabolic pathways have been thoroughly studied [18]. However, there is limited knowledge about the PHA synthesis in psychrophilic and psychrotrophic bacteria from Arctic and Antarctic environment [17,19–21]. Hence, thorough screening and isolation of hyper-PHA producing bacterial strains from polar environments need to be performed to develop PHA polymers for commercial applications and to understand about the significant role of PHA in ecological succession of psychrophilic and psychrotrophic bacteria.

At present, extremophiles from polar environments have been well-recognized for the discovery of novel biomolecules including exopolysaccharides, biosurfactants, and enzymes with various biotechnological potential [22–24]. However, there is no report on the PHA from Arctic glacier fore-field soil bacteria and the structure and biotechnological implications of these PHAs are yet to be identified. In this study, Arctic glacier soil psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620 [25] (isolated from Midtre Lovenbreen, Ny-Ålesund in Svalbard) was used as model strain to produce intracellular PHA copolymer since fluorescent pseudomonads are known to produce large amounts of functionally diverse MCL-PHAs from structurally-related and unrelated carbon sources. In addition, the strain *Pseudomonas* sp. PAMC 28620 has the potential to produce significant amount of extracellular polymers such as exopolysaccharides when grown on glycerol [25]. It has been strongly believed that the synthesis of intracellular and extracellular bacterial polymers from identical carbon sources is an interlinked phenomenon and possibly sharing the similar metabolic pathways [3]. Therefore, this study was intended to explore the capacity of *Pseudomonas* sp. PAMC 28620 to synthesize biotechnologically important intracellular polymer, MCL-PHAs.

The main objective of this study was to produce MCL-PHA copolymer from *Pseudomonas* sp. PAMC 28620 using structurally unrelated carbon sources and statistically optimized medium comprising glycerol as main substrate was utilized for batch fermentation process in lab-scale bioreactor. Further, we systematically characterized the structural components of the PHA copolymer by GC–MS, FTIR and NMR (^{1}H & ^{13}C). In addition, molecular weight, physical and thermal properties were evaluated to highlight its potential application in biomedical and pharmaceutical fields.

2. Material and methods

2.1. Microorganism and culture conditions

The wild-type Arctic glacier fore-field soil psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620 [25] (Polar and Alpine Microbial Collection (PAMC), Korea Polar Research Institute (KPRI), Incheon, South Korea) was used in this study. The strain can able to grow at temperature range from 5 to 30 °C and also grow well on Nutrient agar (Acumedia, Lansing, MI, USA), *Pseudomonas* agar (P2102, Sigma-Aldrich, St. Louis, MO, USA) and Lysogeny Broth (LB) medium (Merck KGaA, Darmstadt, Germany). The strain PAMC 28620 was maintained in LB agar plates at 4 °C and used for all culture experiments.

2.2. Shake-flask fermentation

The initial seed culture was prepared by inoculating 50 mL of sterile LB broth using a single colony of PAMC 28620 and grew it for 72 h in a shaking incubator (Han Beak Scientific Co., HB-201SL) with speed of 150 rpm at 25 °C. About 5% (v/v) of seed culture was inoculated into a 1500 mL shake flask containing 400 mL of sterile PHA production medium-1 (PM-1) such as Difco™ M9 Minimal Salts medium (per liter for 5x, 33.0 g Na_2HPO_4, 15.0 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl) with 2% of D (+)-glucose as sole carbon source. The PM-1 also amended with 1 mL of trace element solution (TES). The TES consisted of (per liter in 0.1 N HCl): 0.86 g $MnSO_4{\cdot}4H_2O$, 0.2 g $ZnSO_4{\cdot}7H_2O$, 0.28 g $CoSO_4{\cdot}7H_2O$, 0.25 g $CuSO_4{\cdot}5H_2O$, 3.6 g $FeSO_4{\cdot}7H_2O$. The pH of the PM-1 was adjusted to 7 ± 0.2 and the strain was cultivated under the culture conditions about at 25 °C and at a shaking speed of 150 rpm for 192 h. About 5 mL of fermented cultured broth was intermittently collected to assess the biomass (as cell dry weight, CDW), PHA yield, and pH change during the fermentation process.

2.3. Optimization of production process parameters

A range of carbon and nitrogen sources were amended to the Difco™ M9 Minimal Salts medium (as defined above). The carbon sources were utilized including D (−)-fructose, D (+)-galactose, D (+)-maltose, starch, and glycerol. The carbon sources were sterilized separately by filtration and added to Difco™ M9 medium at a final concentration of 2% (w/v). Different nitrogen sources including peptone (0.2% w/v), yeast extract (0.2% w/v), casein (0.2% w/v), NH_4NO_3 (0.1% w/v), and $NH_4H_2PO_4$ (0.1% w/v) were also tested for PHA accumulation. Different incubation temperatures (5, 15, 20, 25, 30, and 37 °C) were assessed for the effective PHA accumulation by Arctic strain *Pseudomonas* sp. PAMC 28620. All the experiments were performed in triplicate as one factor at a time approach with constant factors (controls) such as D (+)-glucose (2%, w/v), NH_4Cl (0.5%, w/v), and 25 °C. After growing the bacteria until 192 h (stationary phase), the total biomass (CDW) and PHA productivity were determined. After finding the effective carbon and nitrogen sources using above mentioned classical optimization, response surface methodology (RSM) was conducted to determine the precise concentration of carbon (glycerol) and nitrogen source (yeast extract) to enhance the PHA production. Four variables (glycerol, yeast extract, K_2HPO_4, and KH_2PO_4) were used for RSM to construct a 5-level-4-factorial central composite design (CCD). CCD was developed using the software Design-Expert trail version 10 (State-Ease, Inc., Minneapolis, USA) to elucidate the interactions of variables on the PHA production. An experimental design of 30 experiments (five level of each variable) with six central points was formulated and the experiments were conducted in 250 mL Erlenmeyer flasks containing 50 mL of liquid media prepared according to the design (Table S1) and inoculated with 1% of seed culture (approx.

1.10^9 CFU/mL). The flasks were incubated at 25 °C with 150 rpm. The experiments were performed in triplicate and responses such as biomass and PHA (g/L) were studied at the end of 192 h. The 3-D contour graphs were created to understand the interaction of different factors, and the graphs were used to evaluate the optimized components of the medium which influences the responses [26].

2.4. Batch PHA production in bioreactor

Bioreactor level batch fermentation experiments were conducted in a 1 L bench-top fermenter (FMT ST-D, Fermentec Co., Ltd, South Korea) with working volume of 400 mL of production medium-2A (modified version of PM-2) [25]. The PM-2A was designed according to previous optimization experiments and it comprised of (per liter) 30.0 g glycerol, 3.0 g yeast extract, 5.0 g K_2HPO_4, 3.0 g KH_2PO_4, 10 mL $MgSO_4{\cdot}7H_2O$ (100 mM), and 1 mL TES. The prepared PM-2A was added into the fermenter and the initial pH was adjusted to 7.0 ± 0.2 [25] and then sterilized under ex situ condition. The starter culture was prepared in LB medium and about 5% (v/v) mid-log phase seed culture was inoculated into PM-2A. The agitation speed and temperature was maintained as 150 rpm and 25 °C, respectively [25]. Dissolved oxygen (DO) was adjusted at 100% air saturation during the initial stage fermentation and gas flow (aeration rate) was maintained at 1.0 L min^{-1} throughout the fermentation process [27]. Both DO and pH changes were continuously monitored to find out their influences on the PHA production as critical control factors. The overall fermentation time was 192 h and 10 mL of sample was collected at every 24 h interval to determine the biomass, pH change, DO change, PHA production, glycerol, and ammonium concentrations. Fermenter experiments were carried out in triplicate.

2.5. Analytical procedures

The bacterial growth was determined by measuring the absorbance of optical density (OD) values at 595 nm using a UV/visible spectrophotometer (Sunrise™-Tecan). Further, fermented broth obtained from shake flask and bioreactor, was centrifuged at 8000 × *g* for 20 min. The cell pellet was freeze-dried (lyophilized) and weighed to estimate the total biomass as cell dry weight (CDW). PHA quantity and composition were determined by methanolysis and gas chromatography (GC) using a slight modification of a previously described method [28]. The PHA content (wt%) was determined as the percentage of the ratio of total PHA concentration to biomass. The residual biomass was then estimated as the difference between cell concentrations (CDW) and the PHA content. The residual glycerol concentration in the medium was determined by using high performance liquid chromatography (HPLC) (Agilent) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA), coupled to a refractometer. Ammonium concentration was determined by using phenol-hypochlorite reaction method as reported in previous literature [29].

2.6. PHA extraction and purification

PHA polymer was extracted from the bacterial biomass using a modified version of dispersion of chloroform ($CHCl_3$) and sodium hypochlorite (NaOCl) method [29]. About 1 g of freeze-dried biomass was incubated in a dispersion containing 12% NaOCl (Showa Chemical Co. Ltd.) and $CHCl_3$ (≥99%, Sigma-Aldrich) in a 1:1 ratio, at 37 °C for 12 h under 200 rpm shaking in an orbital shaking incubator. After incubation, the resulting mixture was centrifuged at 3500 × *g* for 10 min. The upper layer was that of NaOCl, intermediate layer contained the bacterial cell debris, and the bottom layer was the $CHCl_3$ which comprising the dissolved PHA polymer. The organic $CHCl_3$ layer was gently collected and then polymer was precipitated by adding $CHCl_3$ layer into 10 vol of ice-cold methyl alcohol (99.5%, Samchun) with continual stirring. The precipitated PHA polymer was further collected by centrifugation at 8000 × *g* for 20 min. The white polymer was then purified by multiple methanol precipitation to remove the impurities. The polymer was dissolved in $CHCl_3$ and gently dispensed in a glass petri dish (ID 15 cm) and $CHCl_3$ was left to vaporize at room temperature for 24 h. The resulting film was then manually collected, weighed, and aged for one week before being characterized.

2.7. Determination of monomer composition by gas chromatography–mass spectrometry (GC–MS)

The PHA monomeric composition was identified by GC–MS analysis of the methanolysed polymer. Methanolysis was carried out according to modified method reported in previous literature [28]. About 10 mg of PHA or bacterial biomass was dissolved in 1 mL of $CHCl_3$ and then 1 mL of methanol/H_2SO_4 (85:15 v/v) mixture was added into the vial and incubated at 100 °C for 120 min, then cooled to room temperature. Around 0.5 mL of ultra-pure water (H_2O) was added and the content was slightly vortexed and the resulting solution was allowed to stand for 10 min. The bottom organic layer was separated and remaining H_2O content was removed by using crystalline sodium sulphate. About 1 μL of methanolyzed sample was automatically injected into the Clarus 680 GC–MS (PerkinElmer, USA) equipped with triple axis detector carrying Elite 5 ms column (30 mm length 0.25 mm internal diameter 0.25 mm film) at a split ratio of 10:1 with column flow 1.0 mL min^{-1}. The injector temperature was set at 280 °C while the oven and column temperatures were programmed as 10 °C for 1 min then increase to 120 °C at 15 °C min^{-1}, hold for 2 min, and increase to 300 °C at 10 °C min^{-1} hold for 15 min. Helium was used as carrier gas at 48.3 mL min^{-1} and 0.41 bar pressure [6]. Mass spectra were acquired at 1250 scan speed using electron impact energy of 70 eV at 200Uc ion-source and 280 °C interface temperatures respectively. Complete instrument control is available through TurboMass™ driver. NIST/EPA/NIH library was used to predict the methylated PHAs and their corresponding mass ion.

2.8. FTIR analysis

The major functional groups which present in the purified PHA polymer were detected by FTIR spectrometer (Tensor 27, Bruker Corporation) at room temperature (25 °C) [25]. A thin PHA film was prepared and scanned between the ranges of 600–4000 cm^{-1} wave number at a speed of 1 μm/min with resolution of 4 cm^{-1}. Total 30 scans were employed with a programmed slit opening 2X and air as reference.

2.9. NMR characterization

The micro-chemical structure of the PHAs produced by Arctic strain PAMC 28620, was investigated using proton (^1H) [30,31] and carbon (^{13}C) NMR analysis [29]. About 20 mg of PHA sample was dissolved in 1 mL of the deuterated chloroform ($CDCl_3$) and further NMR spectra were recorded using a Bruker Avance II 500 spectrometer (Bruker Co., Billerica, MA). The ^1H and ^{13}C spectra were obtained at 500 and 150 MHz, respectively at room temperature. Chemical shifts such as resonance signals (δ) were given in ppm comparative to the outstanding signals of $CDCl_3$ as an internal reference (^1H NMR: 7.26 ppm; ^{13}C NMR: 77.42).

2.10. Molecular weight analysis

The molecular weight of PHA was obtained from GPC (PL-GPC 110, Polymer Laboratories) thermo-stated instrument,

prepared with two mixed-D and one mixed-E PL-gel 5 μm column linked in sequences. The column series were calibrated to 2,580,000–1370 Da using narrow molecular weight polystyrene standards. The PHA sample was dissolved in tetrahydrofuran (THF) at a concentration at 2.0 mg mL^{-1}, filtered through a 0.2 μm filter. About 100 μL of sample was injected into GPC using THF as mobile phase at a flow rate of 1.0 mL min^{-1}. The eluted polymer was detected with a differential refractometer and a multi-angle laser light scattering detector (Dawn Eos, Wyatt, CA, USA), joined in parallel, were used as detectors [13].

2.11. Thermal analysis

DSC experiments were conducted using a TA Instruments Q1000 V9.9 Build 303 (TA, USA) well-found with an auto-cooler. Concisely, PHA sample about 3–5 mg was encapsulated in an aluminum pan and it was chilled from room temperature to −60 °C. Scans were made at a temperature range of −60 to 200 °C at 10 °C min^{-1} and isothermally sustained at 200 °C for 5 min under nitrogen flow rate of 50 mL min^{-1}. Consequently, the sample was quenched to −60 °C and reheated from −60 to 200 °C at 10 °C min^{-1}. The glass transition temperature (T_g), cool crystallization temperature (T_{cc}), melting temperature (T_m), and melting enthalpy fusion (ΔH_m) were taken at the midpoint of the transition, summit of crystal peak, summit of melting peak, and calculated from the area of the endothermic peak in the second heating run, respectively. The percentage of crystallinity (X_p) of the purified PHA was calculated from the heating scans using a formula:

$$Xp = \frac{\Delta Hm}{Wp \times \Delta H^{\circ}m} \quad (1)$$

where ΔH°_m is enthalpy for a theoretical 100% crystalline PHA polymer (146 J g^{-1}) and Wp is ratio of the polymer when using polymer blends [5]. Thermal stability which means the maximum temperature that polymer can withstand before thermal decomposition was studied by TGA using a TA Instrument Q5000 V3.15 Build 263 (TA, USA). Polymer sample was heated from 50 to 900 °C at a rate of 10 °C min^{-1} in an active nitrogen atmosphere at a flow rate of 20 mL min^{-1} [6].

2.12. Statistical analysis

All the experiments were performed in triplicate and the results were analyzed and represented as mean ± standard deviation (SD) (n = 3) using SigmaPlot 10.0. Design Expert Software (State-Ease, Inc., Minneapolis, USA, Trial version 10) was used for polynomial analysis and to plot response surfaces. One-way analysis of variance (ANOVA) was used to estimate the statistical parameters for optimization experiments.

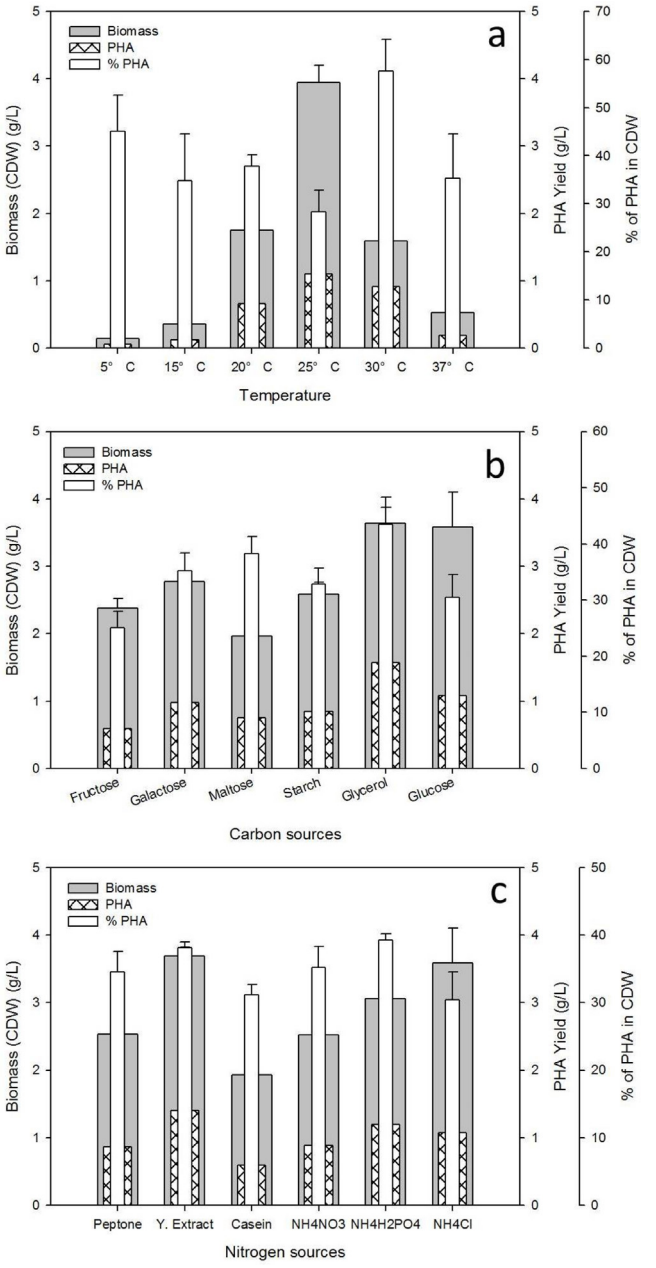

Fig. 1. Environmental and nutritional factors which influence the biosynthesis of MCL-PHAs in *Pseudomonas* sp. PAMC 28620. (a) Effect of incubation temperature on MCL-PHAs copolymer synthesis. (b) Effect of structurally unrelated carbon sources on MCL-PHAs copolymer synthesis and glucose (2%) was maintained as control. (c) Effect of nitrogen sources on MCL-PHAs copolymer synthesis and NH_4Cl (0.5%) was maintained as control.

3. Results and discussion

3.1. Effect of temperature on PHA copolymer synthesis

The effect of different incubation temperature on PHA synthesis has been studied since the bacterium was isolated from Arctic glacier environment. *Pseudomonas* sp. PAMC 28620 was synthesizing significant amount of PHA copolymer in temperature ranging from 5 °C to 37 °C in Difco™ M9 Minimal Salts medium with 2% glucose as sole carbon source (Fig. 1a). The maximum PHA production about 1.10 ± 0.10 g/L was obtained at 25 °C with 28.30 ± 4.53% of PHA in CDW and this percentage is lower than that of other incubation temperatures. The highest percentage of PHA accumulation in CDW was observed at 5 °C and 30 °C with 45.08 ± 7.53% and 57.59 ± 6.51%, respectively. Although, the percentage of PHA accumulation was higher at low and high temperatures, however, the overall biomass and PHA yield was relatively lesser than optimum temperature (25 °C). Moreover, optimum temperature possibly increases the rate of metabolic processes which further enhance the growth of microorganism instead of PHA accumulation. Contrariwise, at low and high temperatures, the bacteria will start to accumulate high amount of PHA as an energy reserve to challenge the physiological stresses induced by fluctuating temperatures. It has been reported that the PHA accumulation can be increased while decreasing the temperature particularly in wild-type and cold-sensitive PHB mutant *Pseudomonas* sp. 14-3 [17]. In this study, Arctic glacier fore-field bacterium *Pseudomonas* sp. PAMC 28620 was reported as an efficient PHA producer and it is

Table 1
PHA copolymer composition as a function of carbon source.

Carbon sources	Relative amount of monomers in purified PHA (%, wt/wt)			
	3HO (C_8)	3HD (C_{10})	3HDD (C_{12})	3HTD (C_{14})
Glucose	11.97	44.65	19.36	24.02
D (−)-Fructose	18.62	52.81	4.77	23.80
D (+)-Galactose	21.63	57.06	2.71	18.70
D (+)-Maltose	12.91	44.55	18.82	23.72
Starch	24.62	55.84	1.55	17.99
Glycerol	32.97	51.39	1.04	14.60

3HO, 3-Hydroxyoctanoate; 3HD, 3-Hydroxydecanoate; 3HDD, 3-Hydroxydodecanoate; 3HTD, 3-Hydroxytetradecanoate.

producing PHA copolymer at low temperature (5–25 °C) and this strain can be used produce PHA in cold environments, e.g. extreme cold countries (especially in winter), wherein the temperature of fermentation process need not be increased to above ambient in order to attain high accumulation of PHA for industrial production. Psychrotrophic bacterial strains including *P. fluorescens* A2a5 [32], *P. extremaustralis* (14-3) [20] and *P. mandelii* CBS-1 [33] have been already reported as potential PHA producers and their ability to grow at low temperatures promotes their significance in biopolymer industries.

3.2. Effect of carbon and nitrogen sources on PHA copolymer synthesis

Precisely how different carbon and nitrogen sources influence the PHA copolymer synthesis was assessed. The structural configuration of PHA synthesized by *Pseudomonas* sp. PAMC 28620 when grown on simple and structurally nonrelated carbon sources at 25 °C for 192 h is shown in Table 1. The effect of carbon sources (D (−)-Fructose, D (+)-galactose, D (+)-maltose, starch, and glycerol) on overall biomass, PHA yield and their corresponding percentage of PHA in CDW are presented in Fig. 1b. We have found that strain PAMC 28620 is capable of synthesizing PHA copolymer using all different carbon sources and highest PHA yield of 1.57 ± 0.07 g/L (43.51 ± 4.79% in CDW) from 3.64 ± 0.24 g/L of biomass when the medium was amended with 2% glycerol. In addition, when glycerol is used as the growth substrate, the molar% of PHA monomer composition is unique with highest ratio of 3HO (32.97%). The yield of PHA copolymer in PAMC 28620 cultivated on unrelated sources can amount to approximately above 25% of the dry biomass. Similar to that of carbon substrates, all nitrogen sources positively exaggerated PHA synthesis when Difco™ M9 Minimal Salts medium with 2% glucose as sole carbon source (Fig. 1c). Yeast extract had the significant effect on biomass (3.68 ± 0.13 g/L) and PHA yield (1.40 ± 0.07 g/L) with 38.08 ± 3.07% PHA accumulation in CDW. In addition, $NH_4H_2PO_4$ has also shown the highest PHA accumulation (39.24 ± 0.94%) in CDW, however, the overall yield of PHA is lesser than yeast extract. Based on these optimization experiments (one factor at a time approach), the modified production medium (PM-2A) was developed with glycerol and yeast extract as the carbon and nitrogen source, respectively.

Biosynthesis of PHA with MCL-saturated fatty acids is an interesting phenomenon of fluorescent *Pseudomonas* sp. PAMC 28620 and this strain perhaps share the ability to accumulate PHA from structurally unrelated simple carbon sources by *de nova* fatty acid synthesis pathway wherein glycerol can be converted to acetyl-CoA as a PHA precursor. Similar kind of phenomenon was also observed in *P. putita* KT2442 [34] and further studies may needed to resolve this hypothesis effectively. The production of MCL-PHAs using glycerol is a relatively cost-effective process when compared to production processes using pure alkanes, structurally related fatty acids or carbohydrates as carbon substrates [12]. In this study, the amount of PHA from the total biomass also rises as the specific growth rate rises, showing a growth associated PHA biosynthesis in this Arctic bacterium. The yield of PHA reached a constant value when specific growth rate became constant at high nitrogen concentrations which is in contrast with the familiar PHA synthesis in many *Pseudomonas* species, where the maximum synthesis is perceived when the specific growth rate of the microorganism is low under nutrient limitation/starvation [35,36].

3.3. PHA copolymer production from Pseudomonas sp. PAMC 28620

Three different batch fermentations such as shake flask experiment with un-optimized medium, shake flask fermentation with statistically optimized medium, and bioreactor fermentation with statistically optimized medium were conducted to study the possibilities of the PHA production from Arctic strain *Pseudomonas* sp. PAMC 28620. The strain PAMC 28620 was able to accumulate the PHA when grown in Erlenmeyer shake flasks containing un-optimized Difco™ M9 Minimal Salts media with glucose as the carbon source. The overall fermentation profiles are presented in Table S2. The dry cell weight increased steadily up to 144 h when the highest biomass was (3.58 ± 0.51 g/L) obtained, after which it decreased to about 2.65 ± 0.12 g/L, at 168 h. The strain had already started to synthesize PHAs by 24 h (early log phase); however, the highest content of PHA accumulation (30.43 ± 4.13% in CDW) has occurred at 144 h (stationary phase). This observation was in agreement with earlier reports of highest PHA synthesis in the stationary phase [13,29]. The maximal yield of PHA was about 1.07 ± 0.13 g/L and the volumetric productivity of PHA was found to be 0.0074 g/L/h, at 144 h of incubation. The pH of the culture medium was decreased as the fermentation progressed and within 144 h the pH had attained 5.82 ± 0.06. A total limitation of nitrogen source NH_4Cl (0.5%) was realized after 96 h of fermentation. The initial glucose concentration was 20 g/L and it was totally consumed by the bacterial cells after 120 h of incubation. In shake flask fermentation, the residual biomass was decreased between 96 and 120 h of fermentation coinciding with a decrease in the ammonium concentration. After the maximum PHA content was achieved, the residual biomass augmented and the PHA content also reduced.

Statistical optimization was performed to predict the better concentration of medium components which involved in the PHA production. The CCD model has produced six central points and the overall second-order polynomial final equation in terms of coded factors for PHA production can be explained as:

$$\begin{aligned}\text{PHA yield (Y)} = {} & +1.83 + 0.22 \times A - 0.12 \times B - 0.075 \times C \\ & + 0.044 \times D - 0.11 \times AB + 0.039 \times AC + 0.021 \times AD \\ & + 0.056 \times BC + 0.10 \times BD - 0.081 \times CD - 0.23 \times A^2 \\ & - 0.14 \times B^2 - 0.21 \times C^2 - 0.22 \times D^2 \end{aligned} \quad (2)$$

Where Y was the response, i.e. PHA yield and A, B, C, D and E were the coded terms for the four test variables. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels of the factors are coded as −1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. In the aspect of PHA yield (Y) A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, and D^2 are significant model terms (Table 2). ANOVA for PHA yield exhibited the model F-value about 1349.64 implies the model is significant at Prob > F-value was <0.0001. There is only a 0.01% chance that a 'Model F-value' this large could happen due to noise. 'Adequate precision' measures the signal (responses) to noise (deviation) ratio. The obtained ratio of 112.196 indicates an

Table 2
ANOVA results obtained from CCD optimization of PHA production by *Pseudomonas* sp. PAMC 28620.

Factors and statistical model	Biomass (cdw)			PHA copolymer yield		
	Mean Square	F Value	p-value Prob > F	Mean Square	F Value	p-value Prob > F
Model	1.43	3806.88	<0.0001	0.39	1349.64	<0.0001
Glycerol (A)	8.95	23914.94	<0.0001	1.17	4067.08	<0.0001
Yeast extract (B)	0.29	787.34	<0.0001	0.33	1135.14	<0.0001
K_2HPO_4 (C)	0.51	1363.13	<0.0001	0.13	469.11	<0.0001
KH_2PO_4 (D)	0.26	695.47	<0.0001	0.04	162.68	<0.0001
AB	0.04	106.82	<0.0001	0.19	657.54	<0.0001
AC	0.06	180.53	<0.0001	0.02	83.48	<0.0001
AD	0.00	0.00E+00	1.0000	0.00	25.11	0.0002
BC	0.12	317.87	<0.0001	0.05	175.92	<0.0001
BD	0.00	8.08	0.0124	0.17	598.47	<0.0001
CD	0.29	764.4	<0.0001	0.11	367.04	<0.0001
A2	6.71	17918.22	<0.0001	1.40	4869.93	<0.0001
B2	0.27	708.3	<0.0001	0.56	1929.77	<0.0001
C2	2.01	5373.04	<0.0001	1.24	4295.48	<0.0001
D2	3.00	8017.41	<0.0001	1.31	4552.13	<0.0001

Prob > F less than 0.0500 indicate model terms are significant.

adequate signal in the case of optimization (media) for PHA production. This model can be used to navigate the design space. The PHA yield R^2 value about 0.999 is slightly closer to 1 showing that the model to be stronger which can better predict the response and model could explain 99% of the variability in the PHA yield. The moderate value of adjusted PHA yield R^2 (0.998) further supported the accuracy of the model. During the RSM experiments, carbon source i.e. glycerol and yeast extract were found to be critical control factor for the maximum synthesis of PHA. Glycerol had shown a positive impact on the biomass and PHA production. In contrast, yeast extract had shown a positive impact on biomass development and negative impact on PHA synthesis. The remaining two factors K_2HPO_4, and KH_2PO_4 had shown the moderate effect on the biomass development and PHA copolymer synthesis. Three dimensional response surface curves were plotted to study the interaction of substrates on the PHA accumulation (Fig. 2). Shake flask experiment with statistically optimized medium, the maximum PHB production about 1.82 ± 0.01 g/L was obtained from the biomass of 4.15 ± 0.01 g/L after 144 h of fermentation. Precisely at this stage, the total PHA content was reached about 44.05 ± 0.30% in CDW with volumetric productivity of 0.012 g/L/h of PHA. Similar to that of previous experiment, pH of the fermented broth was also decreased to 5.59 ± 0.23.

The factors optimized from the shake flask experiments (CCD) were taken into the lab-scale fermenter for the production of PHA by *Pseudomonas* sp. PAMC 28620. Batch fermentation process was conducted to find out the kinetics for the production of PHA in bioreactor. The mean ± SD values of biomass, PHA yield, % of PHA productivity, change of pH, and DO were plotted in Fig. 3. Initially the PHA synthesis was low up to 72 h and then accumulation of PHA was considerably increased as the fermentation progressed (Fig. S1). The maximum PHA content was reached (52.18 ± 4.12%) at 144 h of fermentation and the highest biomass of 7.95 ± 0.66 g/L was obtained with 4.12 ± 0.18 g/L of PHA from strain PAMC 28620. The time of maximum production in fermenter is similar to CCD optimization where we obtained the maximum production at 144 h. The overall volumetric productivity of PHA was determined as 0.028 g/L/h. A gradual decrease in DO and pH of production medium was observed from initial value of 100% and 7.0 ± 0.2 to a minimum of 43.2 ± 1.71% and 5.8 ± 0.09, respectively, at the end of 192 h of fermentation. PHA producers are known to synthesize PHAs when subjected to stressful conditions elicited by limitations of nitrogen, phosphorous, and oxygen [37]. Generally, limitation of DO is one of the most common factors to initiate PHA synthesis in fermenter studies. In this study too, DO was found to be the main limiting factor for synthesis of PHA. This observation completely agrees with the results of previous studies [30,31], where PHA synthesis was induced by the reduced level of DO. Apart from DO, the pH of the medium also known to affect the PHA yield. Therefore, we utilized un-buffered medium to check the PHA production kinetics. In our study, the pH of the culture medium decreased during the growth, from its initial value of 7.0 ± 0.2 to a minimum of 5.82 ± 0.06, 5.59 ± 0.23 and 5.80 ± 0.09 from shake flask (un-optimized & optimized medium) and bioreactor fermentation processes during stationary phase, resulting in inhibition of PHA copolymer degradation. End of logarithmic growth phase

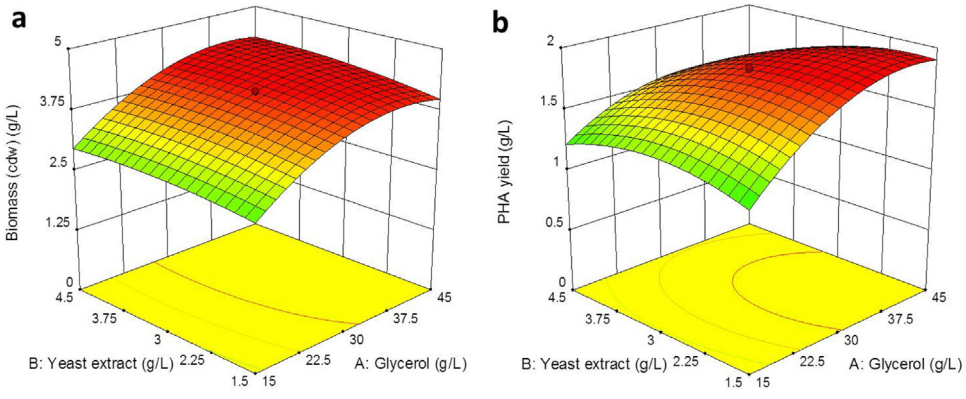

Fig. 2. Three dimensional response surface graphs obtained from CCD statistical optimization show the interactive effects of medium ingredients (glycerol and yeast extract) on the production of biomass (a) and MCL-PHAs copoymer (b) by *Pseudomonas* sp. PAMC 28620.

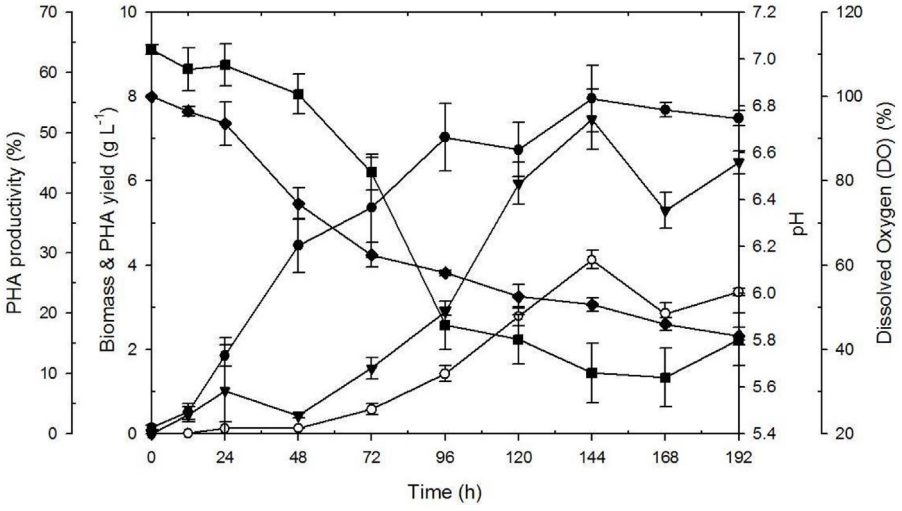

Fig. 3. MCL-PHA copolymer production from *Pseudomonas* sp. PAMC 28620 in lab-scale bioreactor. Biomass (●), MCL-PHAs copolymer yield (○), pH change (■), dissolved oxygen concentration (♦), and MCL-PHAs copolymer accumulation (%) in cell dry weight of the PAMC 28620 (▼) in 3% glycerol enriched production medium (PM-2A). The values shown are means ± SDs from three independent experiments.

overlapped with the decrease of pH and rapid utilization of carbon sources (glucose and glycerol). This reflection was consistent to previous studies where low pH situations have been stated to prevent the consumption of the PHA [30,38]. This observation in the current study is essential for the production of PHA copolymer using *Pseudomonas* sp. PAMC 28620 and other PHA producing fluorescent *Pseudomonas* spp.

After 144 h, the yield of PHA started to decrease and reached about 45.05 ± 1.57% in CDW at 192 h. At the starting of fermentation, the C/N ratio of the PM-2A was 30:3 (g/L); however, the amount of carbon and nitrogen decreased steadily in the PM-2A as the fermentation progressed and by 156 h had almost 80% dropped from the initial ratio. Therefore, the decrease in the PHA yield observed after 144 h could be due to the amount of carbon in the medium had reduced significantly. As a result of this decrease, the strain PAMC 28620 had started to utilize the synthesized PHA as an energy source to sustain its growth and metabolism. The PHA productivity obtained from this study is relatively higher than that of other Antarctic bacterial strains and it strongly establish the industrial prospects of strain PAMC 28620 (Table 3) and also this is the first study describes the scaled-up MCL-PHA copolymer production process in fermenter using a cold-adapted Arctic bacterial strain. Strain PAMC 28620 utilizes glycerol as its best carbon sources; therefore, MCL-PHAs can be produced more economically from PAMC 28620 than other polar strains which requires most expensive carbon sources like sodium octanoate and glucose (Table 3). In addition, strain PAMC 28620 grew to a high concentrated cell density than other polar strains and produce higher yields of MCL-PHA copolymer at a lower cost, which confirms the industrial significance of PAMC 28620.

3.4. PHA polymer monomer composition

The structural configuration of PHA polymer was identified by GC–MS analysis of the 3-hydroxyalkanoate methyl esters prepared by total methanolysis and compared with a standard mixture of bacterial fatty acid methyl esters. GC analysis had shown that the PHA polymer is comprised of four even numbered (carbon) saturated fatty acids as monomers: 3-hydroxyoctanoate (C_8, 3HO), 3-hydroxydecanoate (C_{10}, 3HD), 3-hydroxydodecanoate (C_{12}, 3HDD), and 3-hydroxytetradeconate (C_{14}, 3HTD) with retention time about 3.81, 6.10, 6.80, and 8.47 min, respectively (Fig. S2). PHA monomeric molar ratio (%, wt/wt) was determined as 25.52, 52.12, 5.67, and 16.67% of the 3HO, 3HD, 3HDD, and 3HTD, respectively and their corresponding mass spectrum from the MS library results were displayed in Fig. S3. In this study, the PHA was produced from *Pseudomonas* sp. PAMC 28620 while growing on structurally unrelated carbon source such as glycerol. Based on the GC–MS analysis, it has been confirmed that the polymer produced by Arctic bacterium *Pseudomonas* sp. PAMC 28620 is a PHA copolymer with saturated-MCL fatty acids and 3-hydroxydeconate (3HD) is the predominant monomer of this polymer. This observation was found to be in complete agreement with previous reports in which PHAs copolymers composed of 3HO, 3HD, 3HDD, and 3HTD as the monomeric units [5,6,13,39]. It has been believed that the presence of unsaturated fatty acids may enhance the survival of bacteria in

Table 3
PHA production in bacteria isolated from the Arctic and Antarctic environments.

Microorganisms	Isolated from	Carbon source	PHA productivity (% in CDW)	Reference
Pseudomonas sp. UMAB-40	Casey Station and Signy Island, Antarctica	Sodium octanoate (0.5%)	48%	[19]
		SPKO (0.5%)	40%	
		Glucose (0.5%)	23%	
		Glycerol (0.5%)	11%	
Pseudomonas sp. UMAB-25	Casey Station and Signy Island, Antarctica	Sodium octanoate (0.5%)	15%	[19]
Janthinobacterium sp. UMAB-60 & UMAB-56	Casey Station and Signy Island, Antarctica	Glucose (0.5%)	20%	[19]
Pseudomonas sp. 14-3.	Cierva Point, Danco Coast, Antarctica	Sodium octanoate (15 mM)	70%	[20]
Neptunomonas antarctica CCTCC AB 209086	Nella Fjord, Antarctica	Fructose	26%	[21]
Pseudomonas sp. PAMC 28620	Midtre Lovenbreen, Svalbard, Arctic	Glycerol (3%)	52.18%	This study

SPKO, Saponified palm kernel oil; CDW, Cell dry weight.

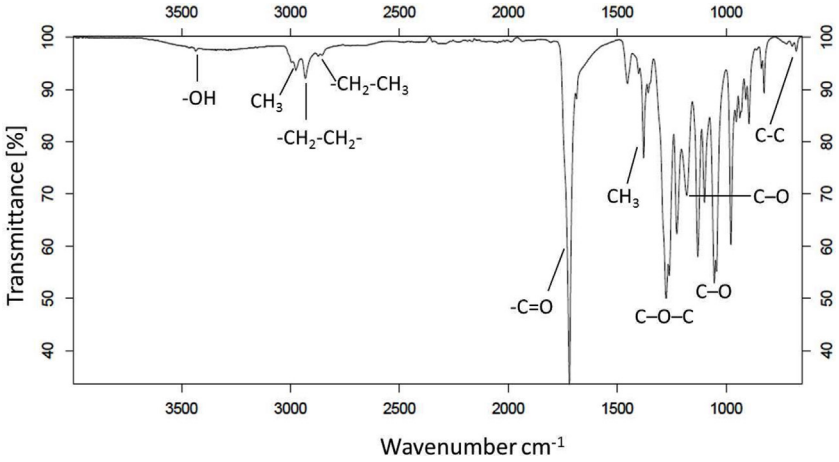

Fig. 4. FTIR spectrum of purified MCL-PHAs copolymer. The spectrum was recorded ranging from 4000 to 650 cm^{-1}. FTIR spectrum was also used to determine the crystallinity index (CI) and the CI for extracted MCL-PHAs copolymer was found to be 0.481.

the extreme cold conditions including Antarctic environment [19]. However, strain PAMC 28620 did not produce any unsaturated form of fatty acids, and our results substantiates with the report of Ayub et al. [20] and they proposed that the synthesis of saturated fatty acids also possibly having a significant role to increase the bacterial survival rate under cold and oxidative stress [17]. Though, further studies are needed to explore the metabolic pathways which involved in the PHA biosynthesis and bacterial stress management especially in Arctic and Antarctic environment.

3.5. PHA polymer functional group analysis

The polymeric functional groups which present in the structure of the PHA were detected by nondestructive attenuated total reflectance FTIR. The principle infrared absorption bands (peaks) were perceived in the intermission of 4000–650 cm^{-1} (Fig. 4). The absorption band at 3435.29 cm^{-1} was assigned to the hydroxyl group of the PHA polymer. The typical spectra of methyl (CH_3) and methylene (CH_2) peaks in the extracted PHA can be detected in the region 3000–2600 cm^{-1}. An absorption peak at 2975.73 cm^{-1} corresponds to the stretching of asymmetrical methyl (CH_3) group. It has been reported that the presence of asymmetric CH_3 can possibly produce union of C—H—O, that is, of hydrogen bridges. Moreover, stretching in the region 2975.73 cm^{-1} also involving in the formation of interactions between CH_3 and carbonyl (C=O) group [5]. Absorption peak at 2932.14 cm^{-1} correspond to the stretching of asymmetrical CH_2 and these functional groups are mainly responsible for the formation of the lateral chains of the monomeric units. The absorption peak at 2855.68 cm^{-1} corresponds to the stretching of symmetrical CH_3 and the strength of this peak is correlated to the conformational change acquired in the process of crystallization [6].

A strong signal at 1719.69 cm^{-1} was assigned to carbonyl (C=O) ester bond stretching vibration [5], which also associated with amorphous region of the polymer and it can be considered as a PHA marker peak. Furthermore, vibration at 1719.69 cm^{-1} also contributes to PHA film maximal crystallinity. The weak signals at 1686.64 and 1453.34 cm^{-1} are belongs to amide I (–CO—N–) carbonyl stretching vibration and N—H bending vibration of amide II, respectively. These amide signals were observed often with PHA polymer due to the intervention of bacterial intracellular components (lower trace) during the PHA extraction process and moreover these protein signals are also described to be species specific [6]. A number of solid peaks at wavenumber values between 1450–1000 cm^{-1} due to CH_3, CH_2 twist, and C—O stretches. A typical terminal CH_3 groups signal was recorded at 1379.05 cm^{-1} and it is quite common feature of PHA. In the region 1300–1000 cm^{-1} appears the stretching of the ethers feature C—O—C linkages. More specifically, a strong vibration at 1275.94 and 1181.31 cm^{-1} were observed due to asymmetric C—O—C and C—O stretching, respectively. Multiple absorption peaks at 1054.85–678.18 cm^{-1} were assigned to C—O and C—C extending pulsation in the amorphous phase. Thus the polymer extracted from *Pseudomonas* sp. PAMC 28620 is confirmed to be PHA.

The FTIR spectrum was also used to calculate the crystallinity of the extracted PHA. A comparative range of the crystallinity was determined by standardizing the absorbance at 1181.31 cm^{-1} to that of 1719.69 cm^{-1} band (δs), which is insensitive to the degree of crystallinity. The peaks at 1181, 1226, and 1275 cm^{-1} are crystallinity-sensitive signals but the band at 1181 cm^{-1} is more specific C—O stretching and well resolved than the others. Thus, a crystallinity index (CI) can be defined as the ratio of the peak intensities at 1181 cm^{-1} to that of 1719 cm^{-1}. In this study, the CI for extracted PHA was found to be 0.481. This CI is not to be confused with an absolute degree of crystallinity but it is valuable as a comparative measure.

3.6. Structural characterization by NMR analysis

The polymer purified from *Pseudomonas* sp. PAMC 28620 was analyzed by NMR to elucidate the structure of the PHA copolymer. The NMR peak assignment was carried out according to previous literatures [5,6,13]. In ^1H NMR, the signals of the multiplets resonance at 2.450–2.771 ppm are assigned to methylene (CH_2) protons (1) of α and β-carbon atoms (Fig. 5a). A very strong signal at 5.237–5.276 ppm corresponds to the methine (methyne) (CH) protons (2) of α and β-carbon atoms. The signal centered at 1.576 ppm is characteristic of methylene (CH_2) protons (3) near to the β-carbon in the side-chains. A clear signal at 1.281 ppm was assigned to the methylene (CH_2) protons (4 to12) of the PHA side-chains. Finally, ^1H spectra had a strong signal at 0.863–0.880 ppm indicating the presence of terminal methyl (CH_3) protons (7, 9, 11, and 13) in β-position of the medium chain monomers. It has been reported as a CH_3 group joined to a chain of four CH_2 groups in 3HO, six CH_2 groups in 3HD, eight CH_2 groups in 3HDD, and ten CH_2 groups for 3HTD [5,6,13]. Based on the proton NMR analysis, the structure of purified PHA was constructed and displayed in Fig. 5b.

The PHA extracted from *Pseudomonas* sp. PAMC 28620 was also analyzed by ^{13}C NMR. Fig. 6 shows the ^{13}C NMR spectrum of PHA produced from glycerol enriched medium. The chemical

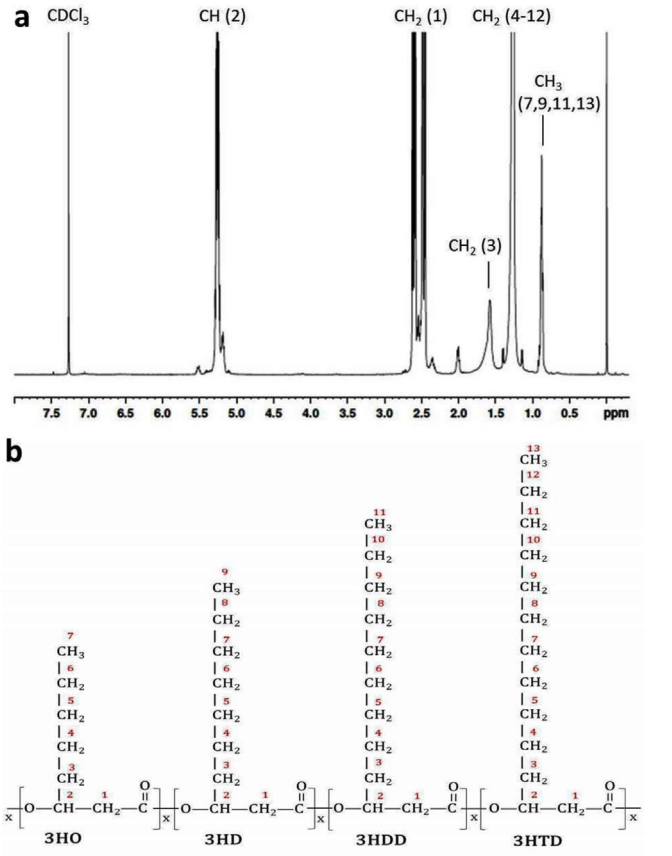

Fig. 5. ^{1}H NMR spectrum (500 MHz) of MCL-PHAs copolymer obtained from *Pseudomonas* sp. PAMC 28620. (a) Proton NMR spectrum was enlarged for peak assignment and protons resonance signals were designated with their corresponding protons. (b) The structure of MCL-PHAs copolymer based on the ^{1}H NMR. The numbers are indicating the proton position in the PHA copolymer. 3HO, 3-Hydroxyoctanoate; 3HD, 3-Hydroxydecanoate; 3HDD, 3-Hydroxydodecanoate; 3HTD, 3-Hydroxytetradecanoate.

Table 4
Chemical shift signals (in ppm) from ^{13}C NMR spectra of PHA copolymer.

Carbon	3-Hydroxyalkanoate monomers			
	3HO (C_8)	3HD (C_{10})	3HDD (C_{12})	3HTD (C_{14})
1	169.29	169.29	169.29	169.29
2	39.41	39.41	39.41	39.41
3	70.99	70.99	70.99	70.99
4	33.96	33.96	33.96	32.05
5	24.86	25.22	25.22	123.09
6	31.67	29.33	29.33	134.01
7	22.65	29.15	29.50	27.55
8	13.95	31.93	29.33	29.57
9		22.78	29.15	29.50
10		14.13	32.05	29.33
11			22.65	29.15
12			14.23	32.05
13				22.65
14				14.23

3HO, 3-Hydroxyoctanoate; 3HD, 3-Hydroxydecanoate; 3HDD, 3-Hydroxydodecanoate; 3HTD, 3-Hydroxytetradecanoate.

shifts were assigned for each carbon resonance signals according to data reported for the MCL-PHAs obtained from *P. putita* KT244 [7], *P. aeruginosa* ATCC 27853 [8] and *P. stutzeri* 1317 [40] grown on different structurally unrelated carbon sources. The major anomeric carbon (C_1) chemical shift was detected at 169.29 ppm for 3HO, 3HD, 3HDD and 3HTD. Similarly, C_2 (39.41 ppm) and C_3 (70.99 ppm) were also sharing the same chemical shift signals for all PHA monomers. The chemical shifts at 13.95, 14.13, 14.23, and 14.23 ppm correspond to the methyl groups of 3HO, 3HD, 3HDD, and 3HTD at positions C_8, C_{10}, C_{12}, and C_{14}, respectively. The unique chemical shifts at 123.09 and 134.01 ppm corresponded to the methylene groups of 3HTD at positions C_5 and C_6, respectively. Strong resonance at 29.33 and 29.25 ppm were assigned to C6 and C7 position of 3HD, respectively. In the same region, some extra signals were also found, that seems to be CH_2 groups of larger PHA monomers, such as 3HDD and 3HTD. The detailed ^{13}C NMR chemical resonance signals and their corresponding carbon positions are represented in Table 4. Finally the strengths of the methylene signals in the region between 10–40 ppm, it was likely to determine

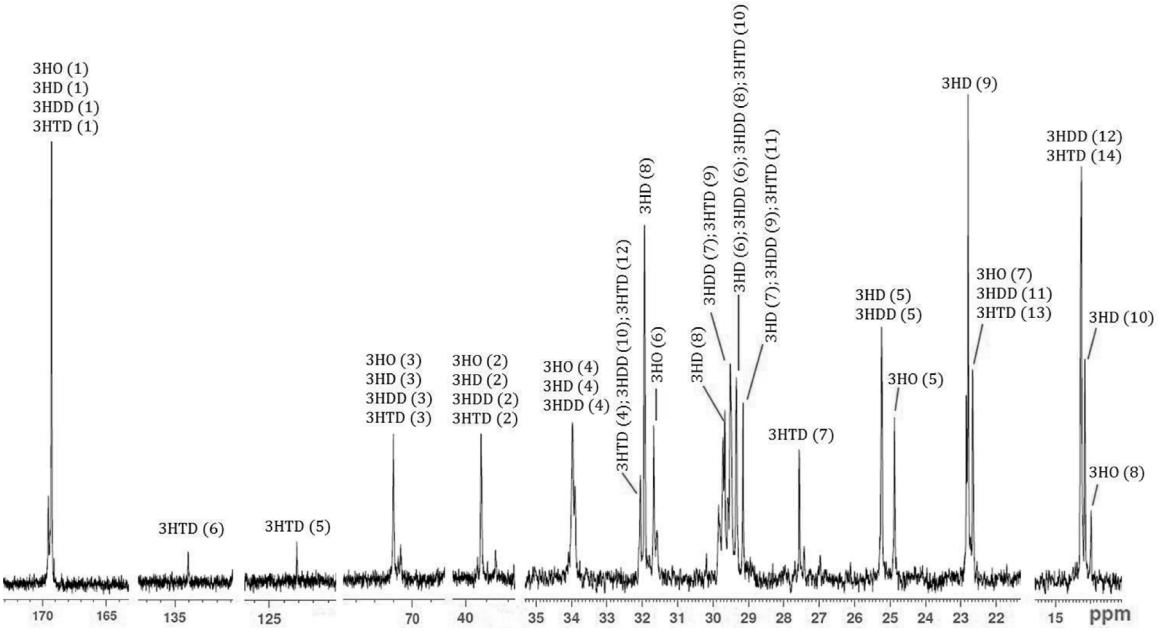

Fig. 6. ^{13}C NMR (150 MHz) spectrum of MCL-PHAs copolymer obtained from *Pseudomonas* sp. PAMC 28620. The coplymeric nature was confirmed and corresponding carbon resonance signals were identified and designated. 3HO, 3-Hydroxyoctanoate; 3HD, 3-Hydroxydecanoate; 3HDD, 3-Hydroxydodecanoate; 3HTD, 3-Hydroxytetradecanoate.

the ratios between all PHA monomers (data not shown). According to ^{1}H and ^{13}C NMR analysis, this polymer was identified as a copolymer consisting of 3HO, 3HD, 3HDD, and 3HTD as monomers and the polymeric composition (ratio) was probably same as we obtained from the GC–MS analysis.

3.7. Molecular weight of the PHA copolymer

The molecular weight and its distribution of the PHA copolymer were determined by GPC. The GPC curve was symmetric; bell shaped and centered at 18.34 min, indicating that the sample was homogenous (Fig. S4). The number-average molecular weight (M_n) and weight-average molecular weight (M_w) were 14754 and 30244 Da, respectively. In addition, Z average (M_z) and Z + 1 average (M_{z+1}) molar mass were determined as 55378 and 84715 Da, respectively. The polydispersity index (PDI) was calculated (PDI = M_w/M_n) as 2.05, more or less similar to that of PDI values of MCL-PHAs biosynthesized by other known *Pseudomonas* spp. [6,39,41]. Although, molar mass of PHA extracted from *Pseudomonas* sp. PAMC 28620 lower than SCL-PHA like PHB [30,31], however, MCL-PHAs have numerous industrial applications such as coating materials, pressure-delicate glues, polymer binding agents in organic solvent-free paints, and also series of biomedical applications, where low molecular weights may be beneficial to the possible applications of the PHA polymer [42]. Future studies will look at the influence of growth conditions and PHA extraction methods on molar mass and PDI value.

3.8. Thermal properties of PHA copolymer

The thermal behavior of PHA copolymer is really crucial for its processing, therefore thermal properties of the extracted polymer was determined by using DSC and TGA. The DSC scan of the purified PHA showed the endothermic melting temperature (T_m) of this PHA copolymer to be 172.84 °C, glass transition (T_g) temperature about 3.99 °C, and apparent melting enthalpy fusion (ΔH_m) was 63.85 J g^{-1} (Fig. S5). In addition, T_g temperature was relatively higher when compared with PHB and other MCL-PHA, which was possibly due to the limited polymer chain mobility. As T_g depicts the conversion of the PHA polymer chains from the systematic arrangement in the amorphous area to the elastic state. The T_m and ΔH_m observed in this study was quite higher due to the presence of long side-chain co-PHA monomer fraction and also side-chain crystallization by the longer side-chain co-monomers may results the high deceptive enthalpy fusion. This observation was found to be in complete agreement with previous reports, especially in *P. putita* KT2442 and *P. putita* Bet001 [5,39,40,43]. DSC can also easily identify the substantial heat release associated with the exothermic crystallization process of PHA. The cool crystallization temperature (T_{cc}) is an essential thermal parameter to describe the crystallization property of elastomeric polymers. A sharp exothermic T_{cc} was observed at 54.61 °C and cool crystallization enthalpy fusion (ΔH_c) of 25.67 J g^{-1} was recorded. The percentage of crystallinity (X_p) was calculated as 43.73% and it was lower than that of the commercial PHB (approx. 60–80%) and also highly comparable with the crystallinity of other PHA copolymers like poly(3-hydroxybytyrate-*co*-3-hydroxyvalerate) (PHB-*co*-PHV) and other PHA blends [40,43,44].

The thermogravemetric configuration had shown that the decomposition of PHA copolymer involves a fast one step-process, and the degradation began at 230 °C with weight of polymer about 99.05% (Fig. S6). The TGA curve demonstrated more than 50, 80, and 90% weight loss at 278.34, 282.66, and 284.25 °C, respectively. This decomposition temperature, which is considerably greater than the melting temperature (T_m), permits an extensive thermal space of workability. The incorporation of increased fraction of longer PHA monomer such as 3HDD and 3HTD might be responsible for the increased thermal stability of this copolymer. In addition, high thermal stability may occur due to unsystematic co-monomer chain-length that leads to a high side chain crystallization, which also provides the thermal stability to the polymer [6,13,45].

The cooling thermogram obtained after the first heating scan at high temperature of 110.93 °C (data not shown) may predict a fast crystallization rate for the PHA polymer. Typically, crystallinity of a polymer is a critical control factor in the degradation of a polymer wherein the amorphous areas in polymers degrade at a much faster rate compared with crystalline areas. The moderate T_m value, a T_g value below 20 °C, and low percentage of crystallinity offers an elastomeric property to this MCL-PHA copolymer. In fact, MCL-PHAs are the only microbial biopolymers, which shows the behavior of thermoplastic elastomers and bear a resemblance to natural elastics (rubber) produced by *Hevea brasiliensis* [46]. Besides, crystallinity is also well-known factor to elicit immune response in vivo [29,47]; therefore the low crystallinity of the MCL-PHAs copolymer produced in this work proposes that it would be an excellent biomaterial for applications in soft tissue engineering, such as cardiovascular, skin and nerve tissue engineering and drug delivery.

4. Conclusion

A PHA copolymer consisting of MCL monomers such as 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) and 3-hydroxytetradecanoate (3HTD) was produced from a psychrotrophic Arctic glacier strain *Pseudomonas* sp. PAMC 28620 when fed with structurally unrelated carbon sources including glycerol. Confirmation of the copolymeric nature of the polymer was carried out using GC–MS and NMR (^{1}H & ^{13}C) analysis. Such a scaled-up production of MCL-PHA copolymer from cold-adapted bacteria has never been reported previously and, therefore, its production could help in better understanding of the properties and implications of PHA in the survival of psychrotrophic and psychrophilic bacteria in Arctic environment. The strain PAMC 28620 has produced about 52.18% MCL-PHA copolymer on cell dry weight basis with great thermal stability, high molecular weight and low-crystalline nature. Therefore, Arctic glacier strain PAMC 28620 can be exploited as an efficient candidate to produce MCL-PHAs and further studies will be focused on the correlation between MCL-PHAs synthesis and bacterial (PAMC 28620) survival under various environmental stresses.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2017.01.053.

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