

Full Length Research paper

Analysis of bacterial strains from contaminated and non-contaminated sites for the production of biopolymers

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A total 18 strains were collected from non-contaminated and contaminated environments, and were purified. All purified strains were characterized for Gram reaction and biochemical analysis. Screening for bioplastic production was done by Sudan black staining. Strains isolated from non-contaminated sites showed no polyhydroxyalkanoate (PHA) production. Biochemical analysis showed that PHA producing strains belong to *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Escherichia* and *Bacillus* genera. PHA extraction was done by sodium hypochlorite digestion method. Strain MS2D showed maximum percentage (65%). Production of PHA was optimized for different temperatures and pH. Selected strains were also tested for exopolysaccharides (EPS) production on EPS detection medium by solvent precipitation method. Four out of nine strains exhibited EPS production ability. EPS production was also optimized for different temperatures and pH.

Key words: Biopolymer, bioplastic, exopolysaccharides, contaminated environment.

INTRODUCTION

The exponential growth of human population has led to accumulation of huge amounts of non-degradable waste materials across our planet. Living conditions in the biosphere are therefore changing dramatically, in such a way that the presence of non-biodegradable residues is affecting the potential special programs leading towards the discovery of new commonly used materials that can be readily eliminated from biosphere, and have designed novel strategies aimed at facilitating the transformation of contaminants (Zinn et al., 2001).

Biopolymers are gaining much more interest in industrial sectors worldwide. The term biopolymers include chemically unrelated products that are synthesized by microorganisms under different environmental conditions (Degeest et al., 2001). Bioplastics and exopolysaccharides are industrially important biopolymers. Bioplastics (polyhydroxyalkanoates) are considered good substitutes for petroleum derived synthetic plastics because of their

similar material properties to synthetic polymers and complete biodegradability after disposal. The main advantage of this type of polymers is that since they are of biological origin, they degrade naturally and completely to CO₂ and water under natural environment by the enzymatic activities of microbes. Bioplastics which are lipid in nature are accumulated as storage materials (in the form of mobile amorphous, liquid granules); allowing microbial survival under stress conditions (Sudesh et al., 2000). The number and size of granules, the monomer composition, macromolecular structure and physio-chemical properties vary, depending upon the organism. The high price of polyhydroxyalkanoates (PHAs) compared to petroleum-based plastics has to be justified by extra advantages. The complete biodegradability of PHAs can justify this higher price for special applications where, e.g. packaging material is a small fraction of the total product price. PHAs are industrially produced by pure cultures using as main substrates glucose and propionic acid. The major costs in the PHA production are determined by the cost of substrate (molasses, glucose and propionate) and extraction of the polymer from the cells (Sudesh et al., 2000).

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The other industrially important biopolymers produced by microorganisms are exopolysaccharides that are secreted by bacteria outside their wall indicated by slime production. These exopolysaccharides are produced by a wide range of microbes but especially by lactic acid bacteria in excess amount so that they can be used industrially for different purposes (Degeest et al., 2001). Extracellularly secreted sugar polymers, or exopolysaccharides (EPS) play an important role in development of texture of yogurts and other fermented milks, low fat cheeses and dairy desserts (Vuyst and Vaningelgem, 2003).

EPS can be subdivided into two groups, the homopolysaccharides, composed of one type of monosaccharide, and the heteropolysaccharides, composed of a repeating unit that contains two or more different monosaccharide (Vuyst et al., 2001). The molecular masses of the EPS from lactic acid bacteria range from 10 kDa and over 200 kDa to > 1,000 kDa (Courtin et al., 2002). Importantly, both structure and molecular mass influence the rheological properties of a polysaccharide (Faber et al., 1998). Also, environmental factors (carbohydrate source, nitrogen source, and carbon/nitrogen ratio of the growth medium) can influence the production, monomer composition, and molecular mass of the EPS produced by a particular strain (Degeest and Vuyst, 1999). The purpose of this study was to analyze the bacterial strains from contaminated and non-contaminated sites for the production of bioplastics and exopolysaccharides.

MATERIALS AND METHODS

Sample collection

Samples were collected from two different sites i.e. non-contaminated and contaminated. Non-contaminated sites were garden soil, infertile garden soil, paddyfield soil, river soil (0 - 4 cm) and river soil (10 cm). While the contaminated sites were molasses and sewage water contaminated soils. The samples were collected in sterilized tubes (Cheesbrough, 2001).

Isolation and purification of bacterial strains

Samples were incubated in nutrient broth for 24 h on shaking. Bacterial load was calculated by serial dilution method. The selected bacterial colonies were purified on nutrient agar plates and subsequently analyzed for gram reaction and biochemical tests (Cheesbrough, 2001).

Screening for PHA producing strains

Screening for PHA producing strains was done by growing the strains in PHA detection medium for 72 h and Sudan black staining was done after every 24 h interval (Arnold et al., 1999).

Estimation of growth curves

Growth curves were analyzed for three selected strains (MS2D, MS2C and FSEWA) for PHA production. These curves were observed for 72 h at 37 °C (Cheesbrough, 2001).

PHA extraction

PHA extraction was done by sodium hypochlorite digestion method after 72 h on PHA detection medium. Cells were collected by centrifugation at 4000 rpm for 15 min. Biomass of 0.2 g was suspended in 5 ml of 0.4% sodium hypochlorite. After 1 h at 37 °C, PHA granules were collected by centrifugation. Palette was washed by acetone and water. The palette was dissolved in chloroform, allowed to evaporate, and PHA weight was noted (Arnold et al., 1999).

Optimization for PHA production

PHA producing bacterial strains was subjected to optimization experiment to calculate the PHA as percentage of biomass. Optimization was done at two different pH (6 and 7) and three temperatures (25, 37 and 45 °C). PHA detection broth was prepared and pH was adjusted to 6 and 7 at fixed carbon to nitrogen ratio (1:1). After inoculation, incubation was given at 3 different temperatures. PHA was extracted after 12 h. Biomass and optical density were taken after 6 h. PHA extraction was done by sodium hypochlorite method (Arnold et al., 1999).

Screening for exopolysaccharide production

Screening for exopolysaccharide production was done by inoculating bacterial growth in exopolysaccharide detection medium for 48 to 72 h. After incubation the exopolysaccharide produced by bacteria was precipitated by adding cold isopropanol in the supernatant of broth. A fine network formed in upper layer of solvent showed exopolysaccharide and that comes to the top of the solvent layer.

Optimization

Optimization was done at different pH (6 and 7) and different temperatures (25, 30 and 37 °C). EPS detection medium was used containing sucrose as carbon source in excess amount. EPS detection was done at every 12 h and EPS was calculated as percentage of biomass. Cell palette was removed after centrifugation. Supernatant was treated with three volumes of isopropanol. The isolates were cultured in 50 ml of medium at 160 rpm for 72 h and then EPS and biomass were calculated accordingly.

RESULTS

Isolation and purification of bacterial strains

A total of 18 strains were isolated from two different sites i.e. non-contaminated and contaminated. From non-contaminated soil samples, 9 strains InGA, InGB, FG1, FG2, PFA, PFB, PFC, RS1 and RS2 were isolated. The highest bacterial load was observed in the paddy field soil (500×10^8 cfu) and lowest in fertile garden (250×10^6 cfu). Whereas from the contaminated environments 9 strains; MS1, MS1, MS1B, MS2A, MS2B, MS2C, MS2D, SEWA and SEWB were isolated. The highest colony forming units was recorded from molasses soil 2 (400×10^4) and lowest in sewage (200×10^5). All the strains were named after sample collection area as infertile garden soil (InG), fertile garden soil (FG), paddy field soil (PF), river soil (RS), molasses contaminated soil (MS) and sewage water contaminated soil (SEW). Gram reac-

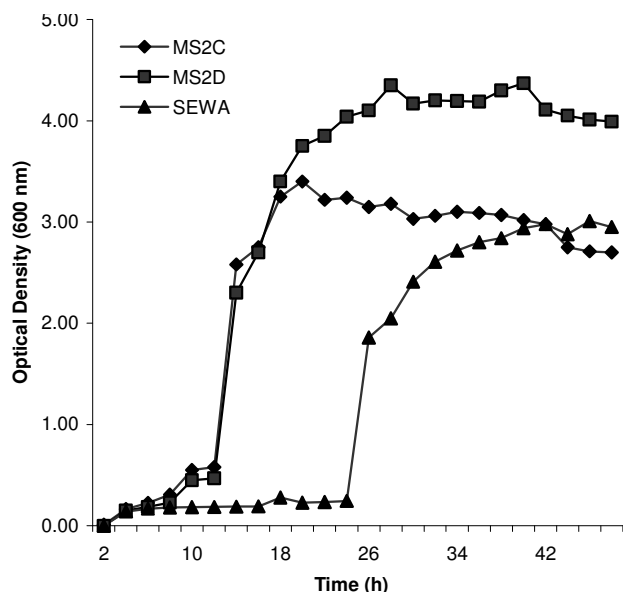


Figure 1. Growth curves of MS2C, MS2D AND SEWA on PHA detection broth (glucose).

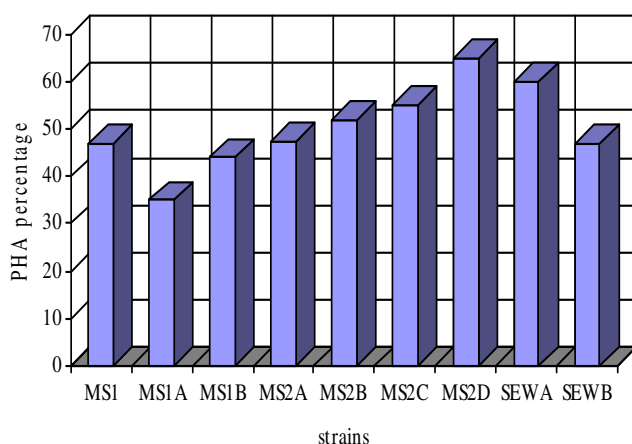


Figure 2. PHA Percentages on PHA detection media (72 h, 37°C).

reaction showed that from all purified strains, 12 were gram negative rods, 2 were gram positive rods and 4 were gram negative coccobacilli. Biochemical analysis showed that PHA producing strains belong to *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Escherichia* and *Bacillus* genera.

Screening for PHA producing strains

None of the 9 bacterial strains isolated from non-contaminated environment showed PHA accumulation and were negative for Sudan black staining. The 9 strains that were selected from contaminated soil were all PHA

producers at varying levels. Sudan black staining was done for every 24 h interval on PHA detection media up to 72 h. All of the strains were positive for Sudan black staining and showed PHA granules. These strains were then subjected to further analysis i.e. biochemical analysis and PHA extraction.

Optimization and PHA extraction

Growth curves were estimated on PHA detection media at 37°C and optical density was taken after 2 h interval to see the growth patterns (Figure 1). PHA extraction was done by sodium hypochlorite digestion method after 72 h on PHA detection medium. Among nine strains three strains produced maximum PHA production i.e. (MS2C, MS2D and SEWA) (Figure 2).

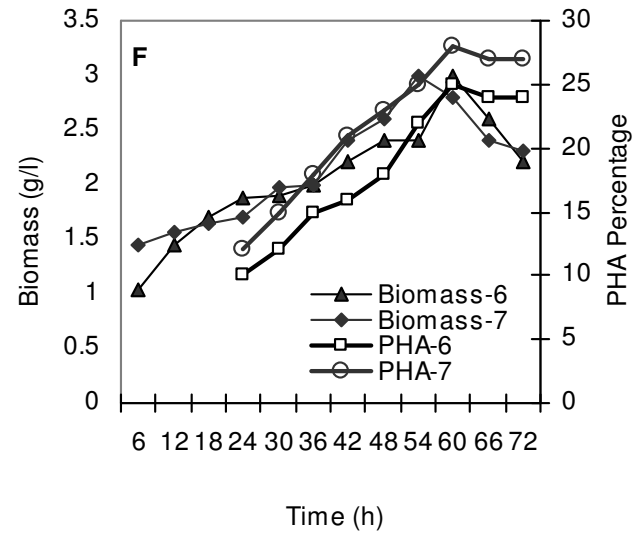
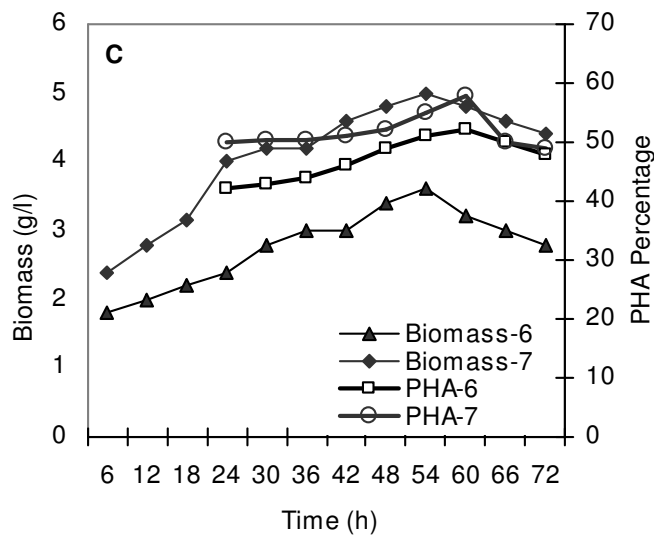
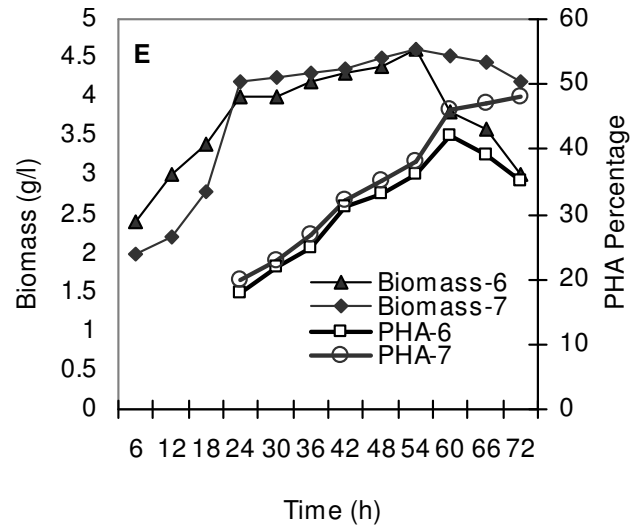
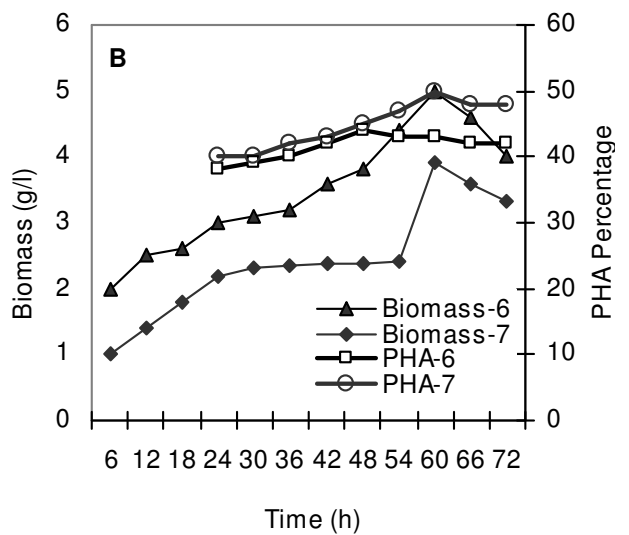
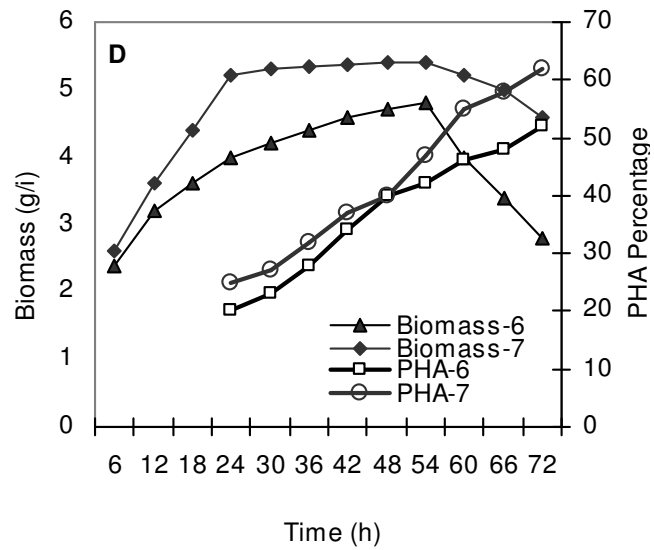
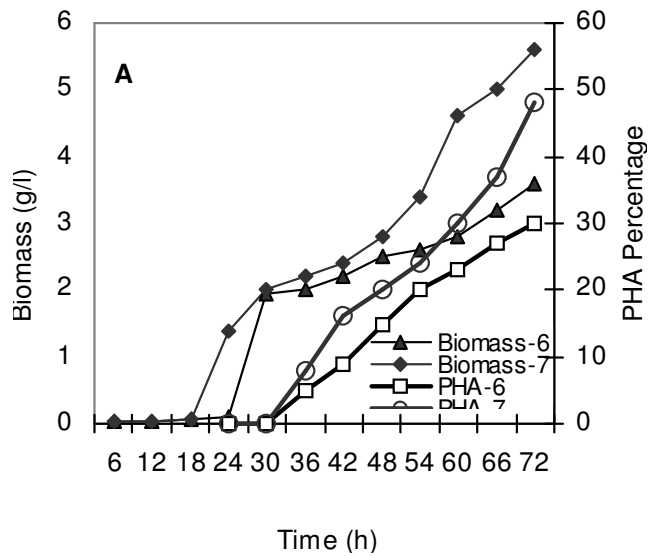
The selection of strains for optimization study was based on the ability to produce PHA. The three selected strains were grown at three different temperatures (25, 37 and 45°C) and two different pH (6.0 and 7.0) in order to analyze PHA productivity. These three strains were grown on PHA detection medium. At pH, 6 and 7 MS2C showed maximum growth at 25°C and 37°C whereas MS2D showed maximum growth at 25°C while SEWA showed maximum growth at 37°C. The strain MS2C showed maximum PHA production (52 and 58%, respectively) at pH 6 and 7 and at 25°C whereas MS2D showed maximum PHA production at 25°C (52 and 65%, respectively). The strain SEWA at pH 6 and 7 showed maximum PHA production (55%) at 37°C (Figure 3 A-I).

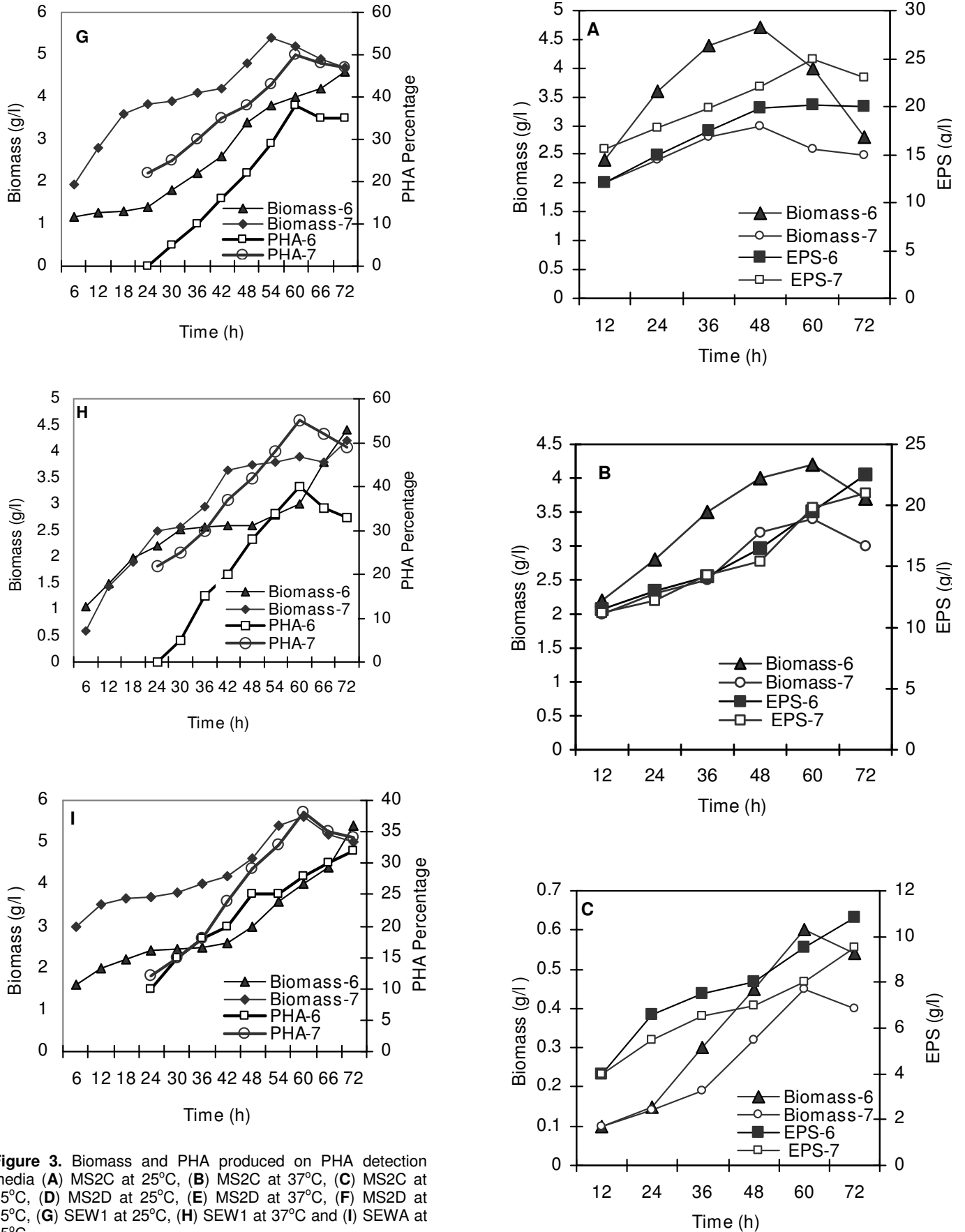
EPS detection and optimization

EPS detection from 9 PHA producing strains was done on EPS detection medium. The 4 strains out of 9 gave positive results (MS1B, MS2B, MS2D and SEWA). The two selected strains (MS2D and SEWA) were grown on EPS detection medium at three different temperatures i.e. 25, 30 and 37°C and pH 6 and 7. MS2D at pH 6 and 7 showed maximum growth and EPS at 25°C with 18 and 25 g/l, respectively. Whereas SEWA at pH 6 and 7 showed maximum EPS at 25°C with 20 and 17 g/l, respectively (Figure 4 A-F).

DISCUSSION

Biopolymers produced by different microorganisms are getting very much importance both in agriculture, economics and health sciences. Among the biopolymers that are industrially important are bioplastics and exopolysaccharides. There is need to optimize the conditions for high production of biopolymers. For this study samples were collected from two different environments i.e. non-contaminated and contaminated. From non-contaminated environment 9 strains were isolated, highest bacterial





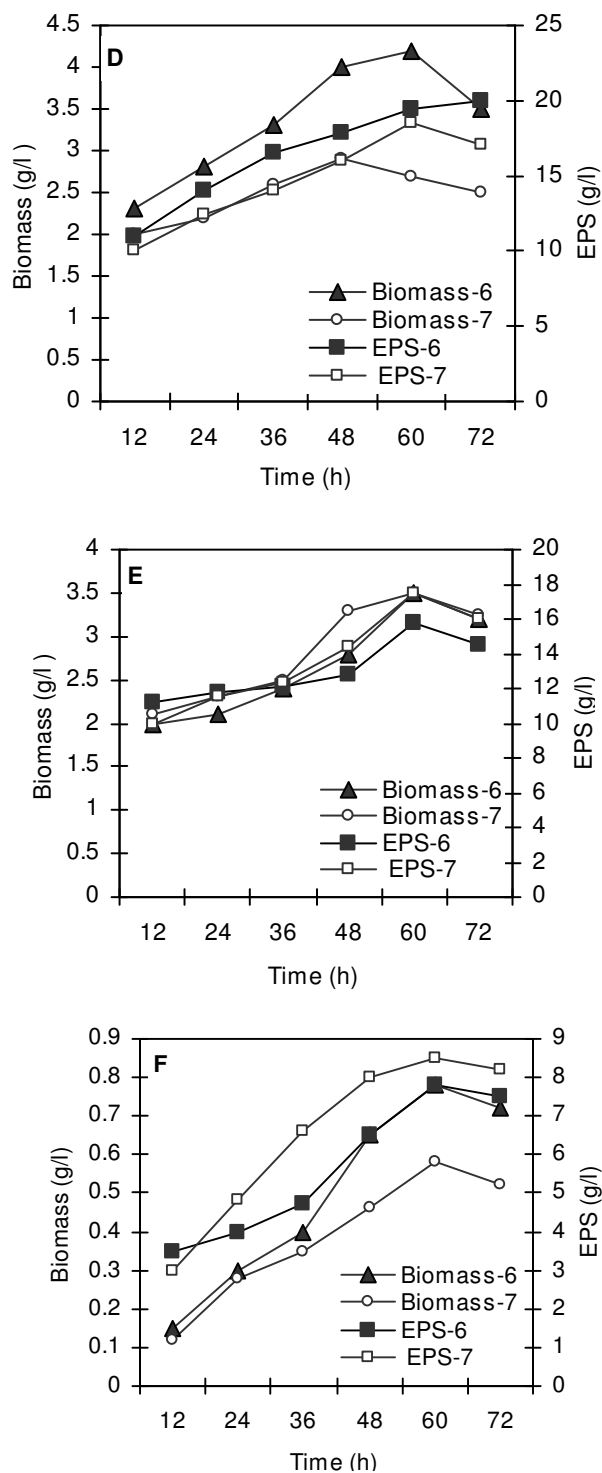


Figure 4. Biomass and EPS produced on EPS detection media. (A) MS2D at 25°C. (B) MS2D at 30°C. (C) MS2D at 37°C. (D) SEWA at 25°C. (E) SEWA at 30°C. (F) SEWA at 37°C.

load was observed in the paddy field soil (500×10^8 cfu) and lowest in fertile garden (250×10^6 cfu). Whereas from the contaminated environments 9 strains were iso-

lated, highest colony forming unit was recorded from molasses soil 2 (400×10^4) and lowest in sewage (200×10^5). A total number of 18 strains were isolated from both sites. The twelve out of 18 strains were gram-negative rods, 2 were Gram-positive rods and 4 were gram negative coccobacilli. All strains were screened for PHA production by Sudan black staining. 9 bacterial strains showed positive results by showing granules in the cells. All bacterial strains from non-contaminated environment showed no granules on Sudan black staining. The bacterial strains MS2C, MS2D and SEWA were strongly positive for Sudan black staining producing granules by all cells after 24 h to peak level after 72 h whereas rest of the strains showed moderate production of granules after 72 h. PHA extraction of all 9 strains isolated from contaminated environments was done by sodium hypochlorite digestion method (Arnold et al., 1999) after 72 h of incubation. Strains (MS2C, MS2D and SEWA) showing strong Sudan black staining, showed maximum percentage of PHA of their biomass (55, 65 and 60%) respectively as compared to all other strains. By the process of screening three strains (MS2C, MS2D and SEWA) were selected for optimization of growth temperatures and pH for PHA production. Based on gram stain's results and biochemical reactions the PHA producing strains belong to the Genus *Pseudomonas*, *Citrobacter* and *Bacillus*.

Growth of the PHA producing bacteria was adversely affected by change in pH and temperature (Huijberts et al., 1992). Bacterial strains (MS2D, MS2C and SEWA) were grown under different pH 6 and 7 and temperatures 25, 37 and 45°C. At 25°C MS2C showed maximum growth at pH 6 and 7, and PHA produced about 60% of biomass after 60 h of incubation growth while at 37°C and with pH 6 and 7 showed relatively low PHA production as compared to 25°C that is, 52%. At 45°C and pH 6.0 PHA production was adversely reduced to 30% of the biomass while at pH 7.0 biomass as well as PHA production increased to 48% of biomass (Figure 3 A-C).

The bacterial strain MS2D on the other hand also produced maximum PHA at 25°C and pH 7.0 i.e. 62% of biomass while at 6.0 PHA production was slightly low i.e. about 51% of biomass at 72 h of growth. MS2D at 37°C and pH 7.0 produced relatively low percentage of PHA as compared to combination of 25°C and pH 7.0, at 37°C and 6.0 PHA productions by MS2D was adversely reduced to 30% of biomass at 72 h of growth. At 45°C, MS2D showed very low production of PHA as well as biomass both at pH 6 and 7 (Figure 3 D-F). The strain SEWA as compared to other two strains showed maximum production of PHA at 37°C and pH 7.0, while at 25 and 45°C PHA production was reduced from 48 to 37% of biomass (Figure 3G-I).

All these observations showed that temperature and pH adversely affected the PHA production and biomass. Optimization of PHA for temperature and pH is necessary for evaluation of optimum growth conditions for maximum PHA production. Alteration in the content of PHA affected

by temperature variance can be due to fact that it might slows down the metabolic activity of microorganisms that ultimately reduces the PHA production ability. Difference in pH also affected the PHA productivity by the strains and it was observed that pH 7.0 and 25°C were favorable conditions for the production of PHA. Pozo et al. (1992) studied effects of culture conditions on PHA production by *Azotobacter* sp. and showed that growth conditions including pH, temperature and carbon source plays an important role in the production rate of PHA.

The PHA production by a particular strain is also related to its biomass. As the biomass increases the bacteria also starts accumulating PHA and produces maximum PHA when its biomass is at its peak level and PHA production is slowed down as the biomass is dropped because at this phase of the growth all the nutrients are depleted leading to decrease in PHA content.

The PHA producing strains were then screened for exopolysaccharides production using EPS detection medium (Tallgren et al., 1999). Four strains out of nine strains produced exopolysaccharides. Strains (MS1B, MS2B, MS2D and SEWA) also produced slime (mucus) on agar plates showed EPS production. Two strains (MS2D and SEWA) were selected for optimization of EPS at different temperatures and pH (25, 30 and 37°C and pH 6 and 7). Two strains were also optimized at three temperatures i.e. 25, 30 and 37°C and two pH values 6.0 and 7.0. MS2D showed maximum production of EPS at low temperature 25°C and pH 7.0, and it is slightly gone down at 30°C although biomass is not so much affected. MS2D at 25°C produced almost 26 g/l of EPS during the time interval of 60 to 72 h. EPS production was drastically reduced at 37°C and is lowered up to almost 11 g/l only from 26 g/l that was produced at 25°C. Tallgren et al. (1999) studied the production of EPS by bacteria and showed that low temperature favors the maximum production of EPS. SEWA produced relatively less EPS than MS2D. SEWA produced 20 g/l of EPS at 25°C, 16 g/l at 30°C and very low production at 37°C i.e. 8.0 g/l only.

The results from both MS2D and SEWA at different temperatures and pH showed that temperature has great impact on the production of EPS (Figure 4 A-F). Low temperature favored the increased production of EPS, the reason being that at low temperature the bacterial growth is slow but a large amount of available sucrose is transformed to polymeric sugars i.e. exopolysaccharides (Tallgren et al., 1999). Production of EPS was also affected by pH but not as much as temperature. Substrate also plays an important role in the production of EPS. Strains that were isolated from sugarcane molasses showed increased production of EPS using sucrose as carbon source. From all the results for optimization of PHA and EPS it is evident that strains, MS2D and SEWA produced PHA as well as exopolysaccharides indicating

that these strains are capable of producing two types of biopolymers.

REFERENCES

- Arnold L, Demain J, Davis E (1999). Polyhydroxyalkanoates. Manual of Microbiology and Biotechnology. Washington, Am Soc. Microbiol. 2: 616-627.
- Cheesbrough M, (2001). District laboratory practice in tropical countries. (2). United Kingdom, University press.
- Courtin P, Monnet V, Rul F (2002). Cell-wall proteinases PrtS PrtB have a different role in *Streptococcus thermophilus/Lactobacillus bulgaricus* mixed cultures in milk. J. Microbiol. 148: 3413-3421.
- Degeest B, Vaningelgem F, Vuyst LD (2001). Microbial physiology, fermentation kinetics process engineering of heteropolysaccharide production by lactic acid bacteria. Int. Dairy J. 11: 747-757.
- Degeest B, Vuyst LD (1999). Indication that the nitrogen source influences both amount size of exopolysaccharides produced by *Streptococcus thermophilus* LY03 modelling of the bacterial growth exopolysaccharide production in a complex medium. Appl. Environ. Microbiol. 65: 2863-2870.
- Faber EJ, Zoon P, Kamerling JP, Vliegthart JFG (1998). The exopolysaccharides produced by *Streptococcus thermophilus* Rs Sts have the same repeating unit but differ in viscosity of their milk cultures. Carbohydr. Res. 310: 269-276.
- Huijberts GNM, Eggink G, Waard PD, Huisman GW, Witholt B (1992). *Pseudomonas putida* KT2442 cultivated on glucose accumulates poly-3-hydroxyalkanoates consisting of saturated unsaturated monomers. Appl. Environ. Microbiol. 58: 536-544.
- Pozo C, Martinez MVT, Rodelas B, Gonzalez LJ (2002). Effects of culture conditions on the production of polyhydroxyalkanoates by *Azotobacter* spp H23 in media containing high concentration of alpechin as carbon source. J. Biotechnol. 97: 125-131.
- Sudesh K, Abe H, Doi Y (2000). Synthesis, structure properties of polyhydroxyalkanoates: biological polyesters. Prog. Polym. Sci. 25: 1503-1555.
- Tallgren AH, Airaksinen U, Weissenberg R V, Ojamo, Kusisto, Leisola M, (1999). Exopolysaccharide producing bacteria from sugar beets. Appl. Environ. Microbiol. pp. 862-864.
- Vuyst DEL, Vin FD, Vaningelgem F, Degeest B (2001). Recent developments in the biosynthesis applications of heteropolysaccharides from lactic acid bacteria. Int. Dairy J. 11: 687-707.
- Vuyst DL, Vaningelgem F (2003). Developing new polysaccharides, p275-320. In B. M. McKenna (eds.), Texture in food, vol. 2. Semi-solid foods. Woodhead Publishing Ltd., Cambridge, United Kingdom.
- Zinn M, Witholt B, Egli T (2001). Occurrence, synthesis medical application of bacterial polyhydroxyalkanoate. Adv. Drug. Rev. 53: 5-21.