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Effect of Nitrogen and Calcium Sources on Growth and Production of PHA of *Pseudomonas* sp. LDC-5 and its Mutant

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Abstract: *Pseudomonas* sp. LDC-5 is an indigenous isolate and its mutant *MNNG-S* produces Short Chain Length (SCL) - Medium Chain Length (MCL) PHAs when grown in unbalanced nutrient condition. Nutrient optimization studies would definitely pave way for large-scale production and there by facilitate commercialization. In this study both the strains were subjected to different nitrogen sources to identify the optimal nitrogen source and the influenze of Calcium chloride supplementation was examined. Mutant strain was found to produce higher and quicker yield compared to wild type amongst the minimal medium studied. Biomass and PHA yield was maximal through the use of Ammonium sulphate as nitrogen source. Calcium chloride supplementation was found to have a positive influence on both strains, in terms of growth rate and production. Time profile studies indicated that cellular biomass and PHA production are unified.

Key words: Media optimization, nitrogen sources, polyhydroxyalkanoates

INTRODUCTION

Many bacteria are capable of accumulating intracellular reserve materials, varying from internal n-alkane pools to polyphosphate (Valappil *et al*., 2007). Among them, the most common one is polyhydroxyalkanoate (PHA), naturally synthesized biodegradable polyester of hydroxyacid stored as carbon reserve (Kumar *et al*., 2009). PHA is classified into three types based on its monomer composition as Short Chain Length (SCL) PHA, Medium Chain Length (MCL) PHA and Long Chain Length (LCL) PHA. PHA consisting of both SCL and MCL monomers has demonstrated a broader spectrum of application properties (Sudesh *et al*., 2000) that makes it appropriate in various applications similar to many conventional synthetic plastics.

P. oleovorans, *P. putida*, *P. citronellolis* and *P. aeruginosa* produce MCL-polyester (Brandl *et al*., 1988) while *Wautersia eutropha* produces SCL PHAs. Majority of PHA producing bacteria accumulates either SCL or MCL PHA, a few have been found to synthesize polyesters containing both SCL -MCL PHAs. As PHAs have properties of thermoplastics and elastomers, it can be moulded, reinforced with inorganic fillers, spun into a fiber, or formed into a film with excellent Gas barrier properties (Hartmann *et al*., 2006; Poirier, 2002). Because of its superior physical and thermal properties (Noda *et al*., 2004), the interest on PHA production hiked up. *Pseudomonas* sp. LDC-5, a strain indigenous to Madurai District was earlier isolated by Sujatha *et al*. (2005a) and its polymer properties were characterized and found to be a SCL-MCL copolymer (Sujatha *et al*., 2005b). Much effort has been consequently devoted towards the reduction in production cost of PHAs. This has necessitated the development of efficient optimized production system that can maximize the yield of PHA. The first step to achieve it would be optimization of media components and fermentation processes.

Accumulation of PHA occurs when the cells become limited for an essential nutrient but have excess of carbon source (Page, 1989). During the initial balanced growth phase, cell mass is produced. Nutrient limitation when imposed in second phase, leads to PHA accumulation (Wang *et al*., 2007). In this study, we investigated the capabilities of *Pseudomonas* sp. LDC-5 and its mutant to produce augmented amount of PHA in presence of nitrogen and Calcium chloride.

MATERIALS AND METHODS

All the analysis was conducted at Lady Doak College, Madurai, India in Department of Zoology and Biotechnology during 2009-2010.

Bacterial strains: *Pseudomonas* sp. LDC-5 originally isolated from soil samples of Madurai district, Tamil Nadu, India and *MNNG-S* (mutant of *Pseudomonas* sp. LDC-5) (Sujatha *et al*., 2005a) were used in this study. The purity of the strain was ascertained by repeated streaking on LB medium. The organisms were grown in

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Basal medium - RC medium (Jan *et al*., 1993). Also, different nitrogen sources (Ammonium sulphate, Amonium chloride and urea) and Calcium chloride were supplemented to study their influenze on cell growth and PHA production at pH 7.0.

PHA production using shake flask: Shake flask cultures were initially carried out in 500 ml flasks containing 200ml of medium. The temperature was maintained at 37ºC, agitation at 300 rpm.

Fermentor studies: Scaling up of PHA production was achieved by using Lab scale fermentor. Seed cultures were inoculated into sterilized media. Fermentation was carried out using 3.5 L Bio console ADI (025) Fermentor (Applikon Biotechnology, Holland) with working volume of 1.0 L. Parameters used for operation: pH (7.0), dissolved oxygen content (30%), agitation speed (3000 g) and temperature (37ºC). The pH was maintained robotically by the addition of 0.5M NaOH and 0.5M HCl. Coconut oil mixed with water (1:1) was used as the anti foaming agent.

Time profile studies: To obtain time profile, during fermentation 50 ml of culture broth was periodically removed for analysis. Cell Dry Weight, PHA yield and Residual cell weight were determined from time to time.

Polymer recovery: Cultured broth was centrifuged at 8000g for10 min and the sediment was washed thoroughly with distilled water. PHA was extracted from the cells as per the method of Sujatha *et al.* (2006). The recovered PHA was subjected to further analysis.

RESULTS AND DISCUSSION

The influence of different nitrogen sources and Calcium chloride on growth and PHA production by *Pseudomonas* sp. LDC-5 (wild type) and its mutant *MNNG-S* were first investigated. Fig. 1 and 2 depicts that biomass and PHA yield was maximized through the use of Ammonium sulphate as nitrogen source supplemented with Calcium chloride in both strains.

Increased yield rate while using synthetic nitrogen sources may be due to the fact that nitrogen served as precursor for vitamins, amino acids, growth factors etc. The amount of PHA accumulated, followed a very similar pattern to its growth, for each of treatment, indicating a growth-related production where the final amount of polymer obtained depends on maximum biomass that are produced. These are in accordance with reports of Bormann *et al.* (1998) and Lageveen *et al.* (1988).

Time profile for wild type strain grown with RC media without any modification depicts that, cellular biomass and PHA are correlated. Lag phase was noticed

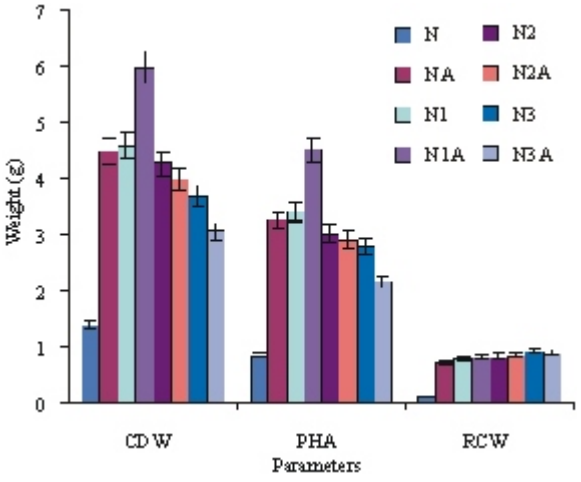

Fig. 1: Effect of different nitrogen sources and supplementation with Calcium chloride on the PHA production per Liter by *Pseudomonas* sp. LDC-5

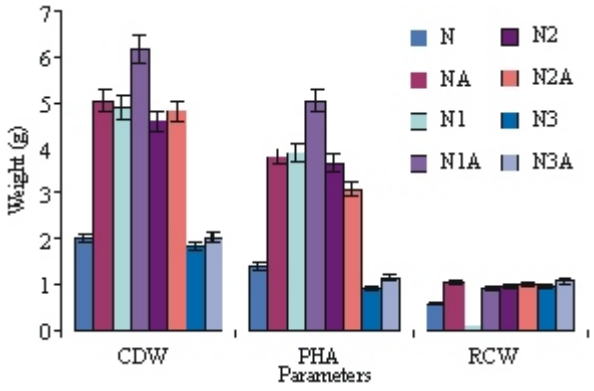

Fig. 2: Effect of different nitrogen sources and supplementation with Calcium chloride on the PHA production per Liter by *MNNG-S*

for a period of 20h. Stationary phase was reached by 40h and maximal PHA was produced at 72 h and by then there was decrease (Fig. 3). The decrease in the PHA yield after 72 h suggest that there is utilization of the storage carbon source - So it is suggestive to harvest at 72 h (Benoit *et al*., 1990; Nam and Ryu, 1985).

For those grown with Ammonium sulphate as nitrogen source and Calcium chloride supplementation, cells have quickly crossed the lag phase and stationary phase was reached sooner (Fig. 4) in comparison with minimal medium. Ammonium sulphate positively influenced growth and PHA production as it is needed by micro-organisms to synthesize all the enzymes that are directly involved and to induce metabolic processes in cell (Choi *et al*., 1999). A similar report for *Alcaligenes latus* was demonstrated by Grothe *et al.* (1999). Nutrient

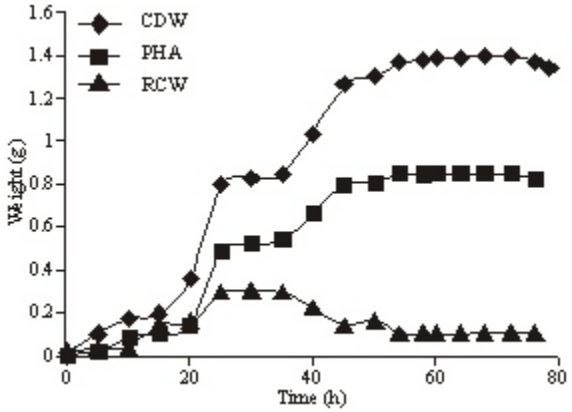

Fig. 3: PHA production per Liter by *Pseudomonas* sp. LDC-5 in basal minimal medium

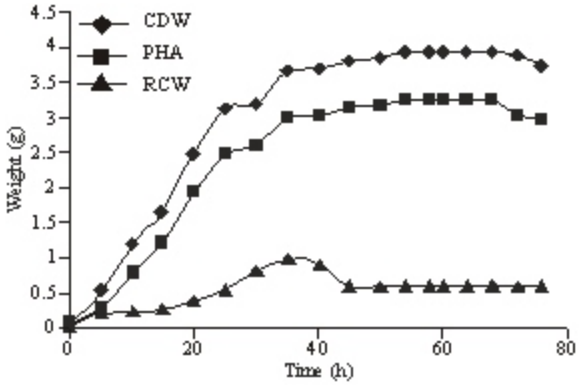

Fig. 4: PHA production per Liter using *Pseudomonas* sp. LDC-5 in media substituted with NH_4SO_4 and $CaCl_2$

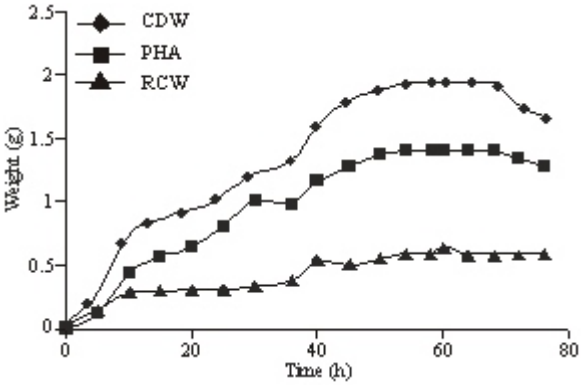

Fig. 5: PHA production per Liter by *MNNG-S* in basal minimal medium

limitation is necessary to trigger PHA production and generally ammonia is used as the critical control factor for uncoupling the growth of cells and PHA production (Wang and Lee, 1997).

PHA production by mutant strain was maximized at 68h after that there was sharp decrease for those grown in

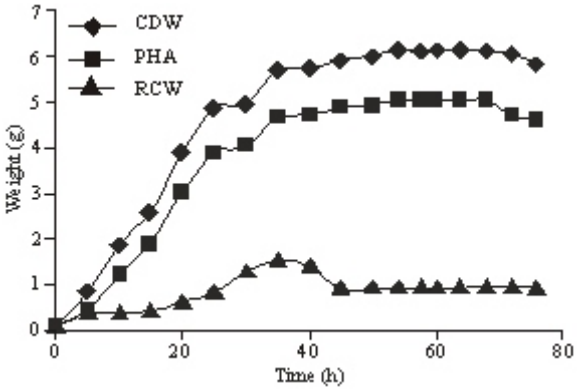

Fig. 6: PHA production per Liter using *MNNG-S* in media substituted with NH_4SO_4 and $CaCl_2$

RC Medium (Fig. 5). This reveals that the optimum time for harvesting PHA from mutant is 68h, which is in turn earlier than that of wild type (72 h). The profile of *MNNG-S* grown with Ammonium sulphate as nitrogen source and with Calcium chloride supplementation was much stabilized (Fig. 6). Stationary phase was reached at 35 h and gradually PHA yield was increasing till 68 h. The yield rate was proportionately higher in mutant strain comparatively with wild type. This may be due to the fact of increased *pha* synthase activity due its mutation in the *phaC* gene.

The supplementation of Calcium chloride had a positive influence in combination with yeast extract and Ammonium sulphate and had a drip with Ammonium chloride and urea for wild type and for mutant Calcium chloride had positive influence on all sources except Ammonium chloride and supplementation of Calcium chloride has led to increased PHA biosynthesis. This could be due to the reality that PHA synthase activity is triggered in presence of Ca^{2+} as per reports of Reusch *et al*. (1986).

This study has recognized Ammonium sulphate as efficient amongst the nitrogen sources and supplementation of Calcium chloride has a positive influence. Further studies on the Physio-chemical properties of the PHA produced by using the altered media composition would pave the way for validating the fact that properties are not negatively affected by the change in growth conditions.

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Abbreviations:

N - Basal Minimum Medium
NA - Basal Minimal Medium supplemented with Calcium chloride
N1 - Ammonium sulphate as nitrogen source
N1A - Ammonium sulphate as nitrogen source supplemented with Calcium chloride
N2 - Ammonium chloride as nitrogen source
N2A - Ammonium chloride as nitrogen source supplemented with Calcium chloride
N3 - Urea as nitrogen source
N3A - Urea as nitrogen source supplemented with Calcium chloride
CDW - Cell Dry Weight
PHA - Polyhydroxalkanoate
RCW - Residual Cell Weight

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