## ABSTRACT

Economic production protocols of biopolymers are desperately required for replacement of petroleum-derived polypropylene polymers that have been extensively used in the society. PHA biopolymers can be naturally synthesized by certain microorganisms such as *Alcaligenes latus*, recombinant *Escherichia coli*, etc., under excess availability of carbon and limiting concentrations of minor nutrients such as nitrogen, potassium, phosphorous, and sulfur. It has been observed that the cost of biopolymers thus produced is relatively high, primarily due to the high cost of agricultural products (sugars, such as glucose, fructose, etc.), which are used as the major substrates during bioreactor cultivation. Recently, it has been reported that Type II *Methanotrophs* such as that *Methylosinus trichosporium*, *Methylobacterium organophillum*, and *Methylocystis hirsuta* can utilize methane gas and have a distinct ability to accumulate PHB based biopolymers.

Experimental protocols were successfully developed for cultivation of *M. organophillum* and *M. trichosporium* using Nitrate Mineral Salt (NMS) media and methanol as substrate. The inoculum propagation protocols for *M. organophillum*, *M. trichosporium,* and *M. hirsuta* were also established in crimp-sealed serum bottles/flasks containing NMS media with an overlay of methane gas, wherein *M. hirsuta* demonstrated the highest biomass production ability, and was therefore, identified as the key organism for in-depth studies in the present investigation. *M. hirsuta* featured 0.08 g/L biomass accumulation after 100 hours cultivation in the serum flasks. Inoculum development protocols for *M. hirsuta* were successfully established in modified Borosil bottles, which facilitated continuous purging of methane with the result of which biomass growth of 2.32 g/l was obtained after 126 h in NMS media. Thereafter, study of batch growth kinetics of *M. hirsuta* was done in

2.5L bioreactor using NMS media under constant methane purging (at 0.05 vvm), which resulted in production of 2.5 g/l biomass with PHB accumulation of 0.55 g/l in 140 hours. To enhance the biomass growth further a fed-batch bioreactor cultivation of *M. hirsuta* was initiated in 3.5L bioreactor wherein sterilized media, (500 ml of concentrated (2.5X NMS) media having reduced (1X) concentration of Potassium nitrate) was supplemented after 200 hours and the overall biomass concentration of 10.57 g/L with PHB accumulation of 1.1 g/L was obtained after 800 hours.

The nutrient concentrations of NMS media were statistically optimized. The concentrations for $KNO_3$, $MgSO_4.7H_2O$, $KH_2PO_4$ and $Na_2HPO_4.12H_2O$ emerged as 1 g/l, 1 g/l, 0.27 g/l and 0.71 g/l, respectively after statistical optimization. The dilutions of NMS media were done to figure out the possibility of improvement of growth rate of *M hirsuta*, wherein it was observed that NMS/4 concentration was most favorable for the maintenance of exponential growth phase of *M. hirsuta* for a longer period of time. *M. hirsuta* was then cultivated in 2L stirred-tank reactor (STR) and 4L airflift reactor (ALR) using 0.25X NMS media with methane purging at 0.05 vvm. This led to biomass and PHB production of 0.59 g/l and 0.16 g/l, respectively, in 74 h in STR and biomass growth of 0.72 g/l and intracellular PHB accumulation of 0.21 g/l after 73 hours for ALR configuration.

Detailed nutrient limitation studies were performed in 2.5L STR and 4L ALR revealed that the limiting concentration of nitrate ions along with constant (non-limiting) availability of methane featured high PHB production within the cells of *M. hirsuta*. The role of inoculum age on the PHB accumulation was identified for *M. hirsuta* cultivation by using the inoculum of different ages (48 & 100 h age) for bioreactor cultivation. It was observed that the culture age of 100h exhibited biomass and PHB productivity of 0.184 $kg.m^{-3}.d^{-1}$ and 0.125 $kg.m^{-3}.d^{-1}$, respectively, which is the

highest productivity reported for *M. hirsuta*.

The batch growth kinetics of *M. hirsuta* using 0.25XNMS media duly purged with methane (at 0.05 vvm) in 2.5L STR and 4L airlift bioreactor were used to develop respective mathematical models, which adequately described the observed batch growth kinetics of *M. hirsuta*. The models were extrapolated to simulate fed-batch cultivation to identify nitrate feeding for maintenance of pseudo steady state of nitrate for enhanced biopolymer accumulation. Upon experimental implementation of the nutrient feeding strategy during the fed-batch cultivations of *M. hirsuta* in STR & air lift bioreactor, it was observed that the developed mathematical model adequately described the experimental batch bioreactor cultivation of *M. hirsuta*. However, the model-predicted maintenance of constant nitrogen concentration during the fed-batch cultivation could not correlate well with the experimentally observed values of biomass and PHB.

Fed-batch cultivation with intermittent (need based) supplementation of NMS media was attempted for cultivation of *M. hirsuta* in 2.5L bioreactor containing 0.25X NMS media duly purged with methane at 0.05 vvm. The NMS media was supplemented in small pulses of 50 ml after 24, 48, 56 and 78 h. The feed concentration was then increased to 50 ml of 2.5X NMS at 150, 175 and 200 hours and 350 ml of 2.5X NMS media was finally added after 228 hours. The above fed-batch cultivation strategy led to biomass production and PHB accumulation of 3.77 g/l and 1.08 g/l respectively.

The co-cultivation of the two organisms (*M. hirsuta* to *M. organophillum*) in NMS-methane media resulted in slightly higher OD (0.65) than the respective pure culture of *M. hirsuta* (OD of 0.59) and *M. organophillum* (OD of 0.06), indicating that co-cultivation of these organisms can result in higher biomass and PHB accumulation.