ABSTRACT

The aim of this work was the study of the regulation of PHA biosynthesis in the thermophilic bacterium *Thermus thermophilus*. The influence of the initial phosphate concentration in polymer production was also investigated. In order to reduce the production cost of PHA in *T.thermophilus* whey, a cheap industrial by product was used as carbon source. Subsequently the extracellular PHB depolymerase of *T. thermophilus* was purified. The physicochemical and catalytic properties of the purified enzyme were studied and its gene was identified. Moreover, it has been shown that *T. thermophilus* is a flagella-motile bacterium. The biochemical properties of flagellin monomers and isolated flagella fibres were studied.

In the first part of the present work the regulation of PHA production in *T. thermophilus* was studied. PHAs are synthesized by several bacterial species and are accumulated as energy or carbon storage materials, usually when a nutritional factor such as nitrogen or phosphorus is limited in the presence of an excess carbon source. In this work the influence of initial phosphate concentration in PHA production in *T. thermophilus* when it was grown in mineral salt media containing sodium gluconate as sole carbon source and excess of nitrogen was investigated. The results indicated that as the initial phosphate concentration in the culture medium was increased from 0 to 25 mM, both the PHA production as well as the enzymic activity of PHA synthase was enhanced. In contrast, when the initial phosphate concentration was increased 50 mM resulted in lower polymer concentration. The results also revealed that the time point of applying nutrient limitation had a significant effect on PHA production. The phosphate limitation in the culture medium containing initially 25 mM was achieved in the begging of stationary growth phase. Since at this time biomass was high enough, the highest PHA concentration, 392 mg/l was obtained from this culture. In contrast, lower phosphate initially concentrations resulted in poor growth and PHA production. In addition it has been shown that as the intracellular ATP levels were decreased the PHA concentration was increased. Hence, the high level of ATP in the culture containing initially 50 mM of phosphate inhibited the biosynthesis of PHA.

The molecular weight of the produced polymer was approximately 280,000 and it was a heteropolymer which was mainly composed of 3-hydroxydecanoate

(3HD) with a molar fraction of 61. In addition, 3-hydroxyoctanoate (3HO), 3-hydroxyvalerate (3HV) and 3-hydroxybutyrate (3HB) occurred as constituents. The melting temperature and crystallization point of the produced polymer were equal to 175.6 ^{o}C and 70 ^{o}C respectively and the degree of crystallinity was approximately 40%.

The ability of *T. thermophilus* HB8 to utilize lactose from whey based media for the biosynthesis of PHAs under nitrogen limitation was also tested. *T. thermophilus* can utilize both, glucose and galactose, the products of lactose hydrolysis. When *T. thermophilus* HB8 was grown in culture media containing 24% (v/v) whey, PHA was accumulated up to 35% (w/w) of its biomass after 24 h of cultivation. The effect of initial phosphate concentration on the PHA production was also investigated. Using an initial phosphate concentration of 50 mM the PHA accumulation was enhanced. Analysis of the produced PHA from *T. thermophilous* HB8 grown in whey-based media revealed a novel heteropolymer consisting of the short chain length 3-hydroxyvalerate (3HV; 38 mol%) and the medium chain length, 3-hydrohyheptanoate (3HHp; 9.89 mol%), 3-hydroxynanoate (3HN; 16.59 mol%) and 3-hydroxyundecanoate (3HU; 35.42 mol%). Despite the low molecular weight of the produced PHA by *T. thermophilus,* whey could be an excellent substrate for the production of heteropolymers with unique properties.

In the second part of this work, *T thermophilus* HB8 has been characterized as a polyhydroxybutyrate (PHB)-degrading microorganism since it grows efficiently and forms clear zones on agar plates containing PHB as sole carbon source. *T. thermophilus* extracellular PHB depolymerase was purified to homogeneity using an affinity chromatography protocol. In order to improve the purification yield, a new chromatography material composed of silica matrix coated with PHB beads was used. The purified enzyme was estimated to have an apparent molecular mass of 42 kDa. The extracellular PHB depolymerase gene was identified as the TTHA0199 of *T. thermophilus* HB8. The amino acid sequence of the TTHA0199 gene product shared significant homologies to other extracellular PHB depolymerases. A catalytic triad consisting of S_{203}, E_{329}, and H_{425} and a pentapeptide sequence (GX_1SX_2G) characteristic for PHB depolymerases (lipase box) and for other serine hydrolases were identified. Purified extracellular PHB depolymerase was stable at high temperatures with an optimum activity at pH 8.0. The apparent *Km* value of the purified enzyme for PHB was 53 μg/ml. As the main product of the enzymic

hydrolysis of PHB, the monomer 3-hydroxybutyrate was identified, suggesting that the enzyme acts principally as an exo-type hydrolase.

In the final part of this work it has been demonstrated that *T. thermophilus* is a flagella-motile bacterium and it showed significant swimming motility in liquid or semisolid media. The production of flagella is enhanced under carbon and nutrient limitation. Flagellin monomer and flagella fibbers were isolated from a culture of *T. thermophilus* growing in mineral salt, sodium gluconate or in rich media. Western blot analysis revealed that flagellin of *T. thermophilus* is a 62 kDa protein, detected intacellularly and extracellularly, indicating that its formation may begin in the cytoplasm as a soluble monomer. Glycan staining of purified flagella and treatment with N-glycosidase F, suggested that flagellin of *T. thermophilus* is a glycosylated protein. The terminal regions of *T. thermophilus* flagellin were sensitive to proteolysis. Proteolytic degradation with trypsin resulted initially in a fragment of 51 kDa and subsequently in a stable fragment of 32 kDa, both detectable with flagellin antibody. N-terminal sequence analysis of *T. thermophilus* extracellular flagellin revealed 100% similarity with flagellins of *Bacillus* sp. and high similarity with flagellins of *Thermotonga* sp.