Original Paper: Microbiology

PROCEEDINGS OF THE LATVIAN ACADEMY OF SCIENCES. Section B, 1994, no. 5/6 (562/563), pp. 89–92.

NITROGEN FIXATION AND POLY-3-HYDROXYBUTYRATE ACCUMULATION BY SOME *AZOTOBACTER* GENUS BACTERIA STRAINS

L. Savenkova*, G. Bonartseva*, Z. Gertsberg*, N. Beskina*, J. Zagreba**, M. Grūbe**

* Institute of Microbiology and Biotechnology, University of Latvia: A. Kirchenšteina ielā 1, Rīga, LV-1067 LATVIA
** A. Kirchenšteins Institute of Microbiology and Virology: A. Kirchenšteina ielā 1, Rīga, LV-1067 LATVIA

The effect of various cultivation conditions on the accumulation of poly-3-hydroxybutyrate (PHB) and nitrogenase activity was investigated in Azotobacter *genus bacteria strains. The main regulation factors of culture growth and PHB accumulation were established to be the aeration level and iron ion concentration in dependence of the nitrogen source. Under the cultivation conditions used, biomass maximum yields for polymer accumulating cultures are higher with low and mean aeration levels. The use of sucrose or fructose as a source of carbon, with an increase of the $FeSO_4 \cdot 7H_2O$ in the medium from 0.005 g/l to 0.05 g/l causes an increase in the biomass by 1.2 to 2.0 times and an increase in the PHB yield. A decrease in the iron ion concentration in the medium results in an increase of oxygen consumption. Introduction of ammonium ions to the medium activates the glutamate dehydrogenase pathway of ammonium assimilation.*

Key words: Azotobacter, *poly-3-hydroxybutyrate, nitrogenase.*

INTRODUCTION

Azotobacter spp. of bacteria typically have a combination of properties such as high nitrogen-fixing and respiratory activities and the ability to accumulate large amounts of reserve carbon compounds and energy in the form of poly-3-hydroxybutyrate (PHB). The process of nitrogen fixation in nitrogen-fixing cells is closely associated with energy metabolism, electron transport and ammonium assimilating pathways. In the *Azotobacter* genus there exists a close association of nitrogen fixation with PHB metabolism.

Most strains of this genus may accumulate up to 10 mg of nitrogen per 1 mg of consumed carbon. This value depends on the strain properties and microorganism cultivation conditions (nutrient medium composition, acidity, temperature, aeration).

Our aim was to study the interrelation of respiratory and nitrogenase activities in *Azotobacter* genus strains which differ in their level of PHB accumulation.

MATERIALS AND METHODS

Azotobacter genus strains from the collection of the A. Kirchenšteins Institute of Microbiology, which differed in PHB accumulation, were used.

Seeding material was cultivated on Ashby medium at a temperature of 28 °C for 48 hours, and modified Berk's medium served as a basis for all liquid nutrient media [4]. Cultivation of microorganisms in liquid nutrient media employed 750 ml Erlenmeyer flasks containing 75 ml, 125 ml and 250 ml nutrient medium, as well as 50 ml bottles containing 20 ml medium. Cultivation was done in a thermal chamber at a temperature of 28 °C on a rotary shaker at 220 rpm.

Nitrogen-fixing activity was determined by the acetylene procedure [7].

The content of main biomass components was determined IR-spectroscopically [1].

RESULTS AND DISCUSSION

A number of *Azotobacter* genus cultures, belonging to various species (*A. chroococcum, A. vinelandii, A. beijerinckii, A. indicum* and *A. agilis*), were tested. Various strains of a single *Azotobacter* species varied widely in the level of reserve polymers. For instance, under similar growth conditions the content of PHB may differ from 0 to 90% of biomass dry weight. Some cultures also produce polysaccharides in significant amounts, reaching 50% dry weight. Two cultures which actively accumulate PHB were also studied (*A. chroococcum* C7 and C8), as well as one which moderately accumulates PHB (*A. vinelandii* V2) and a culture which accumulates the polymer in trace amounts (*A. vinelandii* V1).

Table 1

THE EFFECT OF THE CARBON SOURCE AND CULTURE AGE ON THE CONTENT OF MAJOR COMPONENTS IN THE BIOMASS OF VARIOUS *Azotobacter spp.*

No. of culture	Carbon source, %	Cultivation time, hr	Biomass, mg/ml	Component content, %			
				PHB	protein	lipids	polysaccharides
C7	Glucose	24	2.08	83.4	6.0	3.5	3.2
		48	5.72	81.2	6.2	5.8	1.0
		72	6.14	80.4	6.3	6.9	1.2
	Fructose	24	1.94	87.2	5.5	1.5	1.4
		48	4.75	85.3	5.9	7.3	1.3
		72	6.34	80.2	6.2	10.2	1.3
C8	Glucose	24	0.50	8.5	68.7	3.0	4.2
		48	1.92	80.1	12.2	2.4	2.3
		72	3.72	88.7	4.0	1.5	1.0
	Fructose	24	0.42	14.5	58.4	2.7	2.1
		48	2.48	80.1	12.2	2.3	2.3
		72	3.48	82.2	10.4	3.2	2.8
V1	Glucose	24	0.67	0	58.3	4.5	19.4
		48	1.55	0	62.2	9.7	14.5
		72	1.41	0	51.4	17.5	14.3
	Fructose	24	0.59	0	72.3	2.5	7.5
		48	0.93	2.4	67.1	2.3	19.2
		72	2.39	4.3	63.2	6.3	7.4
V2	Glucose	24	0.61	4.5	71.2	5.4	5.0
		48	1.31	22.3	53.2	4.8	4.2
		72	2.48	29.2	46.5	4.3	3.2
	Fructose	24	0.61	0	72.1	3.2	5.5
		48	1.71	18.0	58.2	2.0	5.3
		72	2.48	21.2	55.4	2.1	5.8

To determine the levels of PHB accumulation and the time period of its formation, we first investigated the growth dynamics and biomass composition of *Azotobacter* strains by cultivation on media with glucose and fructose (Table 1). Intensive PHB accumulation usually starts with the 24th hour of culture growth, which corresponds to the end of the growth logarithmic phase and the beginning of the stationary phase for the growth of periodic cultures of most *Azotobacter* genus strains [2, 10, 13]. The obtained results indicate the maximum levels of the polymer accumulation. The cells of the two active producers, C7 and C8, contained respectively 87.2 and 88.7% PHB. These values are practically equal, but the maximum PHB accumulation in C7 cultures occurs much earlier, with the total yield of the polymer reaching 5 mg/ml, while only 3.42 mg/ml in the C8 strain. The weakly accumulating culture V2 synthesizes up to 29% PHB, and V1 in trace amounts up to 4%. The effect of the carbon source on PHB accumulation and total biomass yield under these cultivation conditions is insignificant.

Oxygen is known as a growth and PHB accumulation regulating factor for *Azotobacter*. The comparative effect of various aeration levels in PHB accumulating and non-accumulating *Azotobacter spp.* strains was investigated. Maximum biomass yields for polymer accumulating cultures were higher with low and medium aeration levels, while no significant differences were observed for the non-accumulating culture V1 (Table 2). The higher biomass yields with low and medium aeration levels are explained by PHB accumulation, since the respiratory activity is enhanced, but the polymer accumulation level is decreased, with high O_2 [5, 9, 12].

Oxygen limitation leads to an increase in the NADH/NAD^+ ratio, inhibiting the tricarbonic acid cycle and thus increasing the intracellular acetyl-CoA level. This in turn increases PHB synthesis [8, 9, 13]. Biosynthesis of large amounts of PHB consumes much NAD(P)H,

Table 2

THE EFFECT OF AERATION LEVEL ON THE GROWTH OF *Azotobacter spp.* BACTERIA IN BURK'S MEDIUM WITH 2% GLUCOSE

No. of culture	Aeration medium, ml	Biomass, mg/ml		
		24 hr	48 hr	72 hr
C7	250	2.06	4.09	5.84
	125	3.05	6.22	5.76
	75	2.64	5.36	4.20
C8	250	0.53	1.59	3.49
	125	0.75	1.75	3.52
	75	1.01	1.70	1.97
V1	250	0.58	0.86	1.22
	125	0.70	1.27	1.54
	75	0.77	1.38	1.57
V2	250	0.45	1.40	2.40
	125	0.61	2.20	3.39
	75	0.30	1.95	2.26

i.e., PHB functions as an electron shunt. This process may compete with nitrogen fixation for reduction equivalents. In case of a lack of an exogenic carbon source and energy, PHB may "release" preserved reduction equivalents required for nitrogen fixation and NADH forming upon polymer catabolism under the effect of transhydrogenase. This restores NADH required for nitrogen fixation. The nitrogen fixing activity of strains C7, C8, V1 and V2 was investigated, in dependence on cultivation conditions. Medium factors, such as carbon source, iron salt concentration and presence or absence of ammonium salt, i.e. factors regulating the culture growth and PHB accumulation, were varied. The attained results (Table 3) showed the following.

1. The presence of ammonium ions in sucrose medium inhibits nitrogenase activity and increases the biomass yield in all cultures investigated. The activity of the ammonium assimilation enzymes may be associated with the activity of nitrogen fixation via product-substrate. Earlier, we established that a low ammonium-pathway of ammonium assimilation (glutamine synthetase/glutamate synthetase) in *Azotobacter* genus cultures practically does not depend on conditions of cultivation. Introduction to the medium of ammonium ions activates a high ammonium-glutamate dehydrogenase pathway of ammonium assimilation, which decreases energy consumption and stimulates synthesis of the main cellular components.

2. A decrease in the iron ion concentration in the medium leads to an enhancement of nitrogen fixation and a simultaneous increase in oxygen consumption (Table 3). These data correlate with the results of electron microscopy. For instance, in the cells of *Azotobacter* strains with low ion concentrations on media with fructose or sucrose, a large number of intracellular membrane formations are observed, which is probably associated with an increase in the amount of membrane-bound enzyme systems of nitrogen fixation and respiration. Hence, iron ions exerting an effect on redox processes in the *Azotobacter* cells, stimulate biological processes such as synthesis of main and reserve biopolymers. The detected regulatory factors of cultivation (iron and ammonium ions and aeration) significantly change the amount of main and reserve biopolymers that can be employed for purposeful PHB production (Table 1).

NAD(P)H, which is required as an electron donor for the nitrogenase reaction in *Azotobacter spp.* and as well for the synthesis of PHB, was shown upon acetyl-CoA reduction to β-oxybutyryl-CoA to form mostly in glucose catabolism by the Enetner-Doudoroff pathway and isocitrate $NADP^+$-dependent oxidation in the Krebs cycle; the latter is considered to be a major NADPH source for the process of nitrogen fixation [12]. However, electron generation *in vivo* for the nitrogenase system reduction has not yet been investigated sufficiently [6]. The major amount of reduced equivalents formed in the Krebs cycle are employed for ATP regeneration by oxidative phosphorylation. The respiratory chain of *A. vinelandii* is branched and possesses three conjugation sites for phosphorylation. The phosphorylation efficiency at these sites is not similar and depends on the oxygen concentration. This permitted Postgate et al. to put forward a hypothesis that the respiratory chain of *Azotobacter* bacteria not only performs the function of regenerating ATP, but also protects nitrogenase from being inhibited by oxygen [11].

Oxygen partial pressure is an important factor regulating nitrogen fixation in the microbial cell. It is assumed [14] that oxygen is capable of inhibiting nitrogenase activity

Table 3

NITROGEN-FIXING ACTIVITY AND CONSUMPTION BY *Azotobacter spp.* STRAINS IN DEPENDENCE ON IRON AND AMMONIUM ION CONCENTRATIONS IN THE CULTURE MEDIUM

No. of culture	Medium			Biomass, mg/ml	Nitrogenase activity nmole/mg d.w.	Oxygen consumption mkMO$_2$/ml d.w.
	Carbon source, 2%	$FeSO_4 \cdot 7H_2O$, g/l	$NH_4H_2PO_4$, 2.0 mM			
V1	Sucrose	0.005	–	1.2	1831.1	167.2
	" "	0.050	–	1.4	1503.0	123.7
	" "	0.050	+	4.4	405.0	47.5
	Glucose	0.050	–	1.4	1588.6	137.2
V2	Sucrose	0.005	–	1.6	1337.6	116.3
	" "	0.050	–	1.8	1189.5	81.9
	" "	0.050	+	3.6	306.2	57.2
	Glucose	0.050	–	1.6	1243.6	119.4
C7	Sucrose	0.005	–	1.4	1615.1	169.7
	" "	0.050	–	1.2	1440.7	137.2
	" "	0.050	+	3.4	356.3	56.9
	Glucose	0.050	–	1.4	1489.7	123.9
C8	Sucrose	0.005	–	1.8	1341.5	114.4
	" "	0.050		2.6	777.3	78.1
	" "	0.050	+	4.0	405.3	53.1
	Glucose	0.050	–	1.6	1319.7	129.4

at four sites: (1) at the site of electron photochemical transport which results in a decrease of reduced flavoprotein directly associated with nitrogenase; (2) at the electron acceptor site where reduced flavoprotein transfers its electrons to nitrogenase; (3) at the site of ATP hydrolysis; and (4) at the substrate-binding centre.

A complete repression of nitrogenase synthesis is observed with increase in oxygen partial pressure (over 0.2 atm for *Azotobacter*). At lower oxygen partial pressures, nitrogenase synthesizes and functions due to the existence in the cell of special mechanisms of protection from oxygen.

REFERENCES

1. Загреба Е., Савенков В., Гиновска М. (1990) ИК-спектрофотометрический контроль химического состава микробной биомассы *Микробная конверсия* (Зинатне, Рига), с. 139–147.
2. Anderson, A. J., Dawes, E. A. (1990) Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalcanoates *Microbiol. Rev.* **54**, No. 4, 450–472.
3. Bergersen, F. J. (1984) Oxygen and the physiology of diazotropic microorganisms *Advances in Nitrogen Fixation Research* Proc. 5th Int. Symp. Nitr. Fix. (Nijhoff/Junk, Netherland).
4. Brotonegro, S. (1976) Nitrogen fixation and nitrogenase activity of *Azotobacter chroococcum*. *Mededelingen Landbauwhgeshool Wageningen, Nederland* **74**, No. 10, 30–48.
5. Carter, I., Dawes, E. (1979) Effect of oxygen concentration and growth rate on glucose metabolism, poly-hydroxybutyrate biosynthesis and respiration of *Azotobacter beijerinckii*. *J. Gen. Microbiol.* **110**, Pt. 2, 393–400.
6. Hager, K. P., Bothe, H. (1987) Reduction of NAD^+ by the reserved respiratory flow in *Azotobacter vinelandii*. *Biochimica et Biophysica Acta* **892**, 213–223.
7. Hardy, R. W. F., Holsten, R. D., Jackon, E. K., Burns R. C. (1968) The acetylene-ethylene assay for nitrogen fixation: laboratory and field evaluations *Plant Physiol.* **43**, 1185–1207.
8. Jackson, F. A., Dawes, E. A. (1976) Regulation of the tricarboxylic acid cycle and poly-3-hydroxybutyrate metabolism in *Azotobacter beijerinckii*, growth under Nitrogen and oxygen limitation *J. Gen. Microbiol.* **97**, 303–312.
9. Laffarty, R. M., Korsatko, B., Korsatko, W. (1989) Microbial production of poly-3-hydroxybutyrate acid *Biotechnology* **6**, 135–176.
10. Page, W. I., Knosp, O. (1989) Hyperproduction of poly-3-hydroxybutyrate during exponential growth of *Azotobacter vinelandii* strain UWD *Appl. Environm. Microbiol.* 55, 1334–1339.
11. Postgate, J. R. (1982) *The Fundamentals of Nitrogen Fixation* (Cambridge Univ. Press).
12. Senior, D. S., Dawes, E. A. (1971) Poly-3-hydroxybutyrate biosynthesis and regulation of glucose metabolism in *Azotobacter beijerinckii*. *Biochem. J.* **128**, 1193–1201.
13. Senior, D. S., Dawes, E. A. (1973) The regulation of poly-3-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. *Biochem. J.* **134**, 225–238.
14. Wong, P. P., Evans, H. J., Klucas, R., Russel, S. (1971) Investigation into the pathway of electron transport to the nitrogenase from nodule bacterioids *Plant and Soil* Spec. Vol. 525–543.

Received October 21, 1993