*Microbiology, Vol. 64, No. 1, 1995, pp. 30 - 33. Translated from Mikrobiologiya, Vol. 64, No. 1, 1995, pp. 40 - 43.*


EXPERIMENTAL ARTICLE

# Influence of Various Oxygen, Sucrose, and Nitrate Concentrations on Poly-β-Oxybutyrate Synthesis in *Rhizobium phaseoli*

**G. A. Bonartseva, V. L. Myshkina, and E. D. Zagreba**

*Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninskii pr. 33, Moscow, 117071 Russia*

Received December 21, 1993

**Abstract** – The conditions for a maximal poly-β-oxybutyrate synthesis in a poorly active strain of *Rhizobium phaseoli* have been optimized by the method of mathematical planning of the experiment. Oxygen, sucrose, and potassium nitrate were used as variable factors. As the result of the experiment, the ratios of the factors investigated have been selected so that poly-β-oxybutyrate content in the strain $A_1$ of *R. phaseoli* reached 80% of dry biomass.

We showed earlier [1] that the chemical nature of carbon and nitrogen sources used is important for poly-β-oxybutyrate (POB) synthesis. POB synthesis can be selectively induced in both inactive (that which is rather common) and active strains of nodule bacteria depending on the carbon and nitrogen sources used. In experiments we used active and inactive strains from clover, alfalfa, and beans. We tested sugars (glucose, arabinose, mannitol, sucrose) and also organic acids (succinic, fumaric, and acetic acids) as the carbon sources. Potassium nitrate, ammonium sulfate, urea, glutamine, glycine, and asparagine were used as nitrogen sources. The best results were obtained with strains of the *R. phaseoli* group grown in the medium with sucrose and nitrate. It is this group of nodule bacteria that is characterized by the greatest capacity for POB synthesis, as pointed out by Salgado *et al.* [7]. Incidentally, the level of POB accumulation in many bacteria is determined not only by the chemical nature of carbon and nitrogen sources, but also by their ratios in the medium. Nitrogen deficiency or the excess of organic substrates results in POB accumulation [8, 11]. It is pointed out in a number of publications that the highest POB level in *Rhizobium* cells is also reached by oxygen limitation [5, 7, 9, 10]. In connection with the possible use of POB in the agriculture and pharmaceutical industries [4, 6], the interest in the search for POB producer strains and in the optimization of growth conditions for a maximal polymer yield is natural [1].

The aim of this work was optimization of conditions for POB overproduction in strains of *R. phaseoli*.

## MATERIALS AND METHODS

*R. phaseoli* cultures – strain $A_1$ (active) and $A_3$ (poorly active) – isolated from soddy-podzolic soil were used in the experiments.

Strains of nodule bacteria were maintained on a pea medium at a pH of 6.8 - 7.0 containing (g/l): peas – 50, sucrose – 5, $K_2HPO_4$ – 0.5, agar – 15. In the experiments, the microorganisms were grown on a synthetic agar medium of the following composition (mg/l): $NaH_2PO_4 \cdot H_2O$ – 150, $CaCl_2 \cdot 2H_2O$ – 150, $MgSO_4 \cdot 7H_2O$ – 250, FeEDTA – 28, $MnSO_4 \cdot H_2O$ – 10, $H_3BO_3$ – 3, $ZnSO_4 \cdot 7H_2O$ – 2, $NaMoO_4 \cdot 2H_2O$ – 0.25, $CuSO_4 \cdot 5H_2O$ – 0.04, $CoCl_2 \cdot 6H_2O$ – 0.025, KI – 0.78, biotin – 0.01, panthothenic acid – 0.1, *p*-aminobenzoic acid – 0.1, folic acid – 0.01, riboflavine – 0.2, $B_1$ – 0.01, $B_6$ – 0.1, $B_{12}$ – 0.02, agar ("Difco") – 1.5%, distilled water, pH 7.0. Concentrations of nitrogen ($KNO_3$) and carbon (sucrose) sources corresponded to the experimental conditions.

The bacteria were grown on Petri dishes. On the surface of the medium in a Petri dish, 0.1 ml of the bacterial suspension with an optical density of 0.5 (measured on FEK-56 M at 550 nm, optical pathway length 1 cm) was placed. To obtain a continuous, steady growth on the agar, inoculation was made with a spatula. The cultures were grown under various oxygen concentrations. For this purpose, desiccators were used. Incubations were carried out in the thermostat at 28°C for 6 days.

Quantitative POB determination was performed by IR-spectrophotometry using the Firordto method [2]. To determine cellular POB content, the bacterial biomass was washed off from the agar medium with 15 ml of tap water, and optical density of the suspension was measured. The cells were then centrifuged and washed twice with sterile tap water. The washed biomass was lyophilized and thoroughly mixed and ground with KBr. Then the tablets were pressed in order to record the infrared spectra. Spectra were taken at IR-20 spectrophotometer (the aperture program was 4, and the rate of registration was 160 $cm^{-1}$/min).

0026-2617/95/6401-0030$12.50 © 1995 МАИК Наука / Interperiodica Publishing

Table 1. Influence of various oxygen, sucrose, and nitrate concentrations on POB synthesis in cells of the active ($A_3$) and poorly active ($A_1$) strains of *R. phaseoli*. Complete factor experiment $2^3$

| Variant | $O_2$, $X_1$ | Sucrose, $X_2$ | $KNO_3$, $X_3$ | POB production, % of dry biomass weight ($y$) | | Regression coefficient ($b_i$) | | Factors and their interaction |
|---|---|---|---|---|---|---|---|---|
| | levels | | | | | | | |
| | +20% −2% | +0.5% −0.2% | +0.075% −0.025% | $A_1$ | $A_3$ | $A_1$ | $A_3$ | |
| 1 | − | − | − | 31.6 | 29.1 | 19.00 | 17.89 | "I" |
| 2 | + | − | − | 23.5 | 17.9 | −1.93 | −3.64 | "$X_1$" |
| 3 | − | + | − | 26.9 | 32.0 | 4.57 | 4.74 | "$X_2$" |
| 4 | + | + | − | 31.2 | 22.0 | −0.05 | −0.14 | "$X_1X_2$" |
| 5 | − | − | + | 1.0 | 4.2 | −9.30 | −7.36 | "$X_3$" |
| 6 | + | − | + | 1.6 | 1.4 | −0.97 | −1.66 | "$X_1X_3$" |
| 7 | − | + | + | 24.2 | 20.8 | 3.83 | 2.99 | "$X_2X_3$" |
| 8 | + | + | + | 12.0 | 15.7 | −3.15 | −0.44 | "$X_1X_2X_3$" |
| 9 average 11% | | 0.35% | 0.05% | 25.4 | 21.9 | | | |

Table 2. Influence of various oxygen, sucrose, and nitrate concentrations on biomass yield in cells of the active ($A_3$) and poorly active ($A_1$) strains of *R. phaseoli*. Complete factor experiment $2^3$

| Variant | $O_2$, $X_1$ | Sucrose, $X_2$ | $KNO_3$, $X_3$ | Biomass yield, mg of dry biomass/ml | | Regression coefficient ($b_i$) | | Factors and their interaction |
|---|---|---|---|---|---|---|---|---|
| | levels | | | | | | | |
| | +20% −2% | +0.5% −0.2% | +0.075% −0.025% | $A_1$ | $A_3$ | $A_1$ | $A_3$ | |
| 1 | − | − | − | 0.48 | 0.35 | 0.65 | 0.45 | "I" |
| 2 | + | − | − | 0.48 | 0.25 | −0.04 | −0.05 | "$X_1$" |
| 3 | − | + | − | 0.60 | 0.35 | 0.18 | 0.12 | "$X_2$" |
| 4 | + | + | − | 0.76 | 0.35 | −0.02 | −0.09 | "$X_1X_2$" |
| 5 | − | − | + | 0.51 | 0.41 | 0.07 | 0.14 | "$X_3$" |
| 6 | + | − | + | 0.41 | 0.35 | −0.08 | −0.02 | "$X_1X_3$" |
| 7 | − | + | + | 1.17 | 0.92 | 0.08 | 0.09 | "$X_2X_3$" |
| 8 | + | + | + | 0.76 | 0.69 | −0.06 | −0.03 | "$X_1X_2X_3$" |
| 9 average 11% | | 0.35% | 0.05% | 0.86 | 0.60 | | | |

Optimization of the medium for POB synthesis was carried out using the method of mathematical planning of the experiment.

## RESULTS AND DISCUSSION

Oxygen, carbon (sucrose), and nitrogen ($KNO_3$) sources were used as factors affecting the process of POB accumulation in bacterial cells. A complete factor experiment (CFE) was run using three variables at two concentration levels (Table 1): $X_1$ – oxygen (+20%, –2%), $X_2$ – sucrose (+0.5%, –0.2%), $X_3$ – $KNO_3$ (+0.075%, –0.025%). Table 1 shows the arrangement of the factor experiment and the process output ($y$), POB accumulation in cells of the active and poorly active strains of *R. phaseoli*. Calculation of regression coefficients makes it possible to estimate both the separate influence of each particular factor on the process and the interaction between factors. From the table, it is seen that the influence of oxygen, sucrose, and potassium nitrate on POB accumulation is almost the same in cells of both strains. Oxygen has a small, but statistically reliable, negative effect on POB accumulation; however, an increase of the oxygen concentration in the medium has a more substantial effect in the strain $A_3$ (POB production decreases more significantly). $X_2$ (sucrose) has an identical positive effect on both strains, i.e., an increase of sucrose concentration in the medium favors POB accumulation in both strains. $X_3$ ($KNO_3$) has a strong negative effect on both strains,

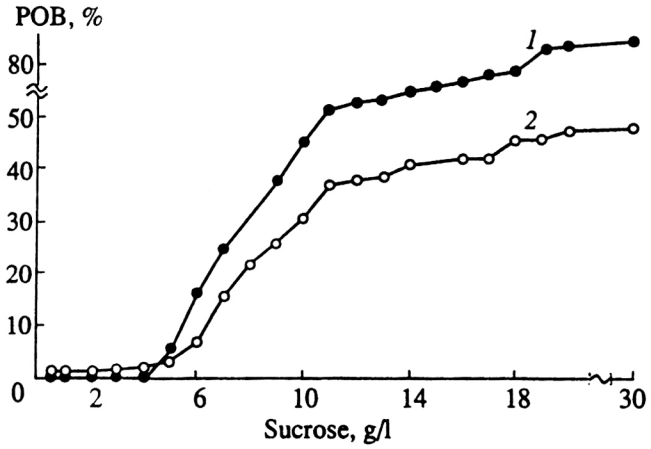


**Fig. 1.** POB content (% of dry biomass weight) in cells of *R. phaseoli* versus the gradient of sucrose concentrations in the medium. (*1*) Poorly active strain $A_1$, (*2*) active strain $A_3$.

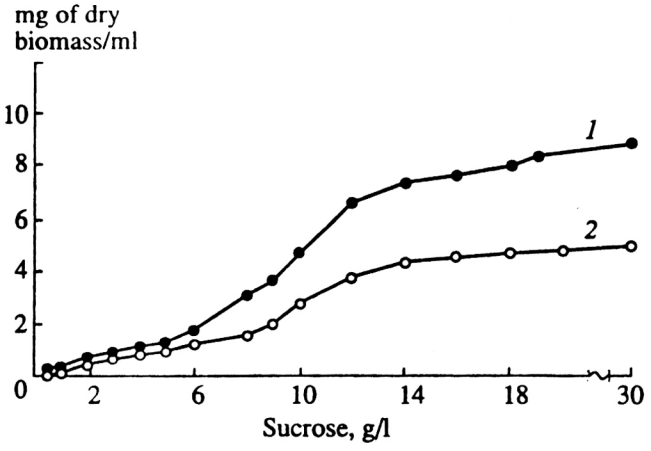


**Fig. 2.** Biomass yield in *R. phaseoli* versus the gradient of sucrose concentrations in the medium. (*1*) Strain $A_1$, (*2*) strain $A_3$.

although this effect is stronger in the poorly active strain $A_1$. A decrease of potassium nitrate in the medium promotes a significant increase of POB content to a greater extent in the poorly active strain. Thus, a maximal POB accumulation in the range of the given concentrations of the factors studied is mediated by a small lowering of oxygen concentration, a rise in sucrose level, and a significant decrease of the potassium nitrate level in the medium.

A maximal POB content is observed in the poorly active strain at C : N ratios of 20 : 1 and 8 : 1 at two given oxygen levels (2%, 20%). In the active strain it is observed at the lower oxygen level (2%) only at a C : N ratio of 20 : 1.

A minimal POB content in both strains is observed at a C : N ratio of 3 : 1, and oxygen does not play any substantial role in this case.

In the system under investigation (Table 1), a reliable positive interaction between a positive factor $X_2$ (sucrose) and a negative factor $X_3$ (nitrate) has been revealed. As it is known, with a positive interaction between negative and positive factors, the effect of the negative factor is diminished when the level of the positive one increases, and the effect of the positive factor is enhanced when the level of the negative one increases [3]. Therefore, an increase of sucrose concentration in the medium may lead to enhanced POB synthesis when the medium contains rather high nitrate concentrations. This may be significant for the elaboration of an industrial technology for POB production, since not only the capability of the industrial strain for oversynthesis of the product but also a high biomass yield from the fermenter are important for production, which cannot be achieved in nodule bacteria at low $NO_3^-$ concentrations in the medium.

A maximal biomass yield in both strains is observed in variant 7 (see Table 2) at minimal oxygen ($X_1$) and maximal sucrose ($X_2$) and nitrate ($X_3$) levels. In this case, the yield value far exceeds the biomass yield in other cases. The experimental results obtained are confirmed by the calculation of regression coefficients ($b_i$) of the factor experiment: two positive factors, $X_2$ (sucrose) and $X_3$ (nitrates), are present in the medium and their interaction $X_2X_3$ is positive. This is true for both strains.

Carbon and nitrate concentrations used in CFE were low; their low level was necessary for a theoretical elucidation of the C : N ratio required for a maximal POB synthesis. Therefore, we employed the increased sucrose concentration gradient in the medium to achieve a maximal intracellular POB accumulation. On the basis of the CFE $2^3$ results obtained, we used the gradient of increased sucrose concentrations in the medium at fixed potassium nitrate and oxygen levels, thus setting various C : N ratios in the medium and using high concentrations of the carbon source to achieve a maximal biomass yield (Figs. 1 and 2). The results obtained completely supported the CFE results. A high POB level was achieved at a C : N ratio of 10 : 1 and was stabilized up to a C : N ratio of 20 : 1 and higher. It is worth noting that active strains of nodule bacteria accumulate POB (Fig. 1) at very low carbon substrate levels in the nutrient medium; at the same concentrations, poorly active strains do not produce POB. At the same time, at high concentrations of carbon substrate in the medium, cellular POB content in poorly active strains is significantly higher than in active strains. A maximal POB content in the poorly active strain of *R. phaseoli* $A_1$ is as much as 80% of the dry cell weight. The biomass yield in the poorly active strain is much greater than the biomass yield in the active strain (Fig. 2) under these conditions.

## REFERENCES


1. Bonartseva, G.A., Myshkina, V.L., and Zagreba, E.D., *Mikrobiologiya*, 1994, vol. 63, no. 1, p. 78.
2. Zagreba, E.D., Savenkov, V.V., Ginovska, M.K., and Yakobson, Yu.O., *Mikrobnaya Konversiya* (Microbial Conversion), Riga: Zinatne, 1990.
3. Maksimov, V.N. and Fedorov, V.D., *Primenenie Metodov Matematicheskogo Planirovaniya Eksperimenta* (Application of the Mathematical Planning Method), Moscow: Mos. Gos. Univ., 1969, p. 121.
4. Braunegg, G. and Korneti, L., *Biotechnol. Lett.*, 1984, vol. 6, no. 12, p. 825.
5. Encarnacion, S., Willms, K., and Mora, J., Program and Abstracts, *9th Int. Congr. on Nitrogen Fixation*, Cancun, Mexico, 1992, p. 502.
6. Lafferty, R., Braunegg, G., Korneti, L., *et al.*, *Proc. III Eur. Congr. Biotechnol.*, Munchen, 1984, vol. 1, p. 521.
7. Salgado, M., Mora, Y., Leija, A., *et al.*, Program and Abstracts, *9th Int. Congr. on Nitrogen Fixation*, Cancun, Mexico, 1992, p. 161.
8. Tal, S. and Okon, J., *Can. J. Microbiol.*, 1985, vol. 31, no. 7, p. 608.
9. Tombolini, R. and Nuti, N.P., *FEMS Microbiol. Lett.*, 1989, vol. 60, p. 299.
10. Vries, W., Stam, H., Duys, J.G., *et al.*, Antonie van Leeuwenhoek, 1986, vol. 52, p. 85.
11. Zevenhuizen, L.P.T.M., Antonie van Leeuwenhoek, 1981, vol. 47, p. 481.