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CONDITIONS FOR PRODUCTION OF EXTRACELLULAR PROTEIN BY BACILLUS BREVIS 47

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ABSTRACT

Bacillus brevis 47 was grown in shake flasks under varying conditions of agitation, culture volume, inoculum size, medium supplementation, and initial pH. It was found to have a high demand for oxygen, and, under appropriate conditions, could produce up to 9 g/l of two extracellular wall proteins in 24 h.

INTRODUCTION

Bacillus brevis 47 is a soil organism first isolated in 1976 (Udaka, 1976). When cultured in a complex medium, B. brevis 47 has been found to secrete up to 5 g/l protein (Udaka, 1976), which can be increased to 12 g/l by supplementation with additional nutrients, particularly glycine and isoleucine (Miyashiro, et al., 1980). The secreted protein is comprised of a 150 kd protein and a 130 kd protein, which form two hexagonally arrayed layers on the inner peptidoglycan cell wall (Tsuboi, et al., 1982). These two protein layers are sloughed off into the medium during the exponential phase of growth, and continue to be secreted into the medium during stationary phase (Yamada, et al., 1981).

The genes for the 130 kd and 150 kd proteins have been cloned and sequenced (Tsukagoshi, et al., 1984; Tsuboi, et al., 1986; Yamagata, et al., 1987; Tsuboi, et al., 1988), and form one transcriptional unit (Tsuboi, et al., 1986).

The present work was aimed at identifying, and in some cases confirming, the culture conditions which promote the highest levels of protein excretion in wild-type cells of B. brevis 47.

MATERIALS AND METHODS

i) Growth Conditions. B. brevis 47 cells were grown in T_2+ medium, which contains 1.0% polypeptone, 0.5% beef extract, 0.2% yeast extract, 1.0% glucose, 0.5% isoleucine, 0.25% glycine, 0.2% $(NH_4)_2SO_4$, 0.03% KH_2PO_4, 0.07% K_2HPO_4, and 0.02% $MgSO_4.7H_2O$ (Miyashiro, et al., 1980). Cultures were grown at 34°C in 125 ml Erlenmeyer flasks on gyratory shakers. Other conditions were as outlined in the Results and Discussion.

ii) Analytical Procedures. Optical density measurements at 600 nm were used as a measurement of cell growth.

Before the measurement of glucose and protein levels, the cells were removed from one ml samples of culture by centrifugation at 5000 x g for 5 min. After the medium was diluted 40X, the glucose remaining was measured using the DNS (dinitrosalicylic acid) test of Miller (1959). Protein was precipitated from the medium with the addition of a 0.1 volume of 50% TCA (trichloroacetic acid). After recovery of the protein by centrifugation at 10,000 x g for 5 min, it was dissolved in 0.01 N NaOH. The amount of protein was measured using the Bradford assay (Bradford, 1976) (Bio-Rad Protein Assay Kit I) with bovine gamma globulin as a standard.

Wall proteins still adhering to the cells were removed by resuspending cell pellets from the one ml samples in 0.5 ml of 50 nM Tris HCl, pH 8.5, and incubating them for one hour at 37°C (Takahashi, et al., 1983). These proteins were recovered and the quantities measured as described above.

Polyacrylamide gel electrophoresis was done according to the method of Laemmli (1970), using a Biorad Protean Dual Slab Gel System.

RESULTS AND DISCUSSION

Cells of B. brevis 47 were grown overnight and the protein isolated from the medium as described above. Polyacrylamide gel electrophoresis confirmed that this strain was producing the 150 and 130 kd proteins.

i) Effect of Aeration. Previous work suggested that B. brevis 47 has a high oxygen requirement for protein production (Miyashiro, et al., 1980). Table 1 shows the effect on cell growth, glucose consumption, and protein production of cultivating cells in three different volumes of culture medium (10, 25, or 50 ml in a 125 ml flask) at 100 or 200 rpm for 24 h. Cultures

Table 1: The effect of the volume of the medium and the speed of shaking on cells of B. brevis 47 in 24 h

speed (rpm)	volume (ml)	OD_{600}	glucose used (g/l)	protein produced (g/l)
100	10	2.490	1.75	1.75
	25	1.473	1.37	0.81
	50	0.559	1.25	0.03
200	10	2.820	2.37	2.03
	25	2.464	1.75	1.38
	50	1.802	1.50	0.75

grown in the smallest volume of medium and shaken at the higher speed were found to produce the highest amounts of protein, confirming that high levels of aeration promote protein secretion by this organism. All subsequent shake flask experiments were conducted by culturing cells in 10 ml of medium in a 125 ml flask at 200 rpm.

ii) Effect of Inoculum Size. Previous reports (Miyashiro, et al., 1980; Tsuchida, et al., 1980) had indicated the use of various inoculum sizes ranging from a loopful of vegetative cells to 5% (v/v). Our initial work with shake flasks and a 2% (v/v) inoculum from an overnight culture had revealed some variability in the results, and, occasionally, a lack of growth. We, therefore, investigated the effect of using inoculum sizes of 1%, 2%, 5%, or 10% (v/v) in shake flasks, and found that an inoculum size of 5% resulted in consistently good growth and protein production (>3 g/l). All subsequent experiments used this inoculum size.

iii) Effect of Medium Supplementation. Under culture conditions identical to those which were reported to have yielded 12 g/l extracellular protein by B. brevis 47 (Miyashiro, et al., 1980), we were consistently unable to obtain more than 6-7 g/l. After 24 h of cultivation, the number of viable

Table 2: The effect of phosphate levels on growth and protein production by cells of B. brevis 47 in 24 h

amount of phosphate (%)	OD_{600}	protein produced (g/l)
0.1	2.817	3.22
0.2	2.768	4.63
0.4	2.649	4.94
1.0	2.230	5.31

cells remained constant (10^9 - 10^{10} cells/ml) even in the presence of greater than 60% of the original glucose supplied. Under these conditions, the cells were still viable, as they doubled when introduced into fresh medium; however, the spent medium was unable to support growth when it was inoculated with fresh cells. This suggested the depletion of a critical component of the medium or the accumulation of an inhibitor.

The addition to the medium of twice the usual amount of beef extract (10 g/l), polypeptone (20 g/l), or yeast extract (8 g/l) had no discernable effect on growth or protein production. The use of phosphate levels of 0.1% (original medium level), 0.2%, 0.4% or 1.0% (w/v), indicated slightly better protein production at the higher level of phosphate, with slightly reduced growth (Table 2). Thus, the above medium supplementation was unable to increase protein production to the levels previously reported.

During these experiments it was noted that the pH of the medium at the end of 24 h was always 8.5, regardless of the extent of protein production, glucose consumption, etc. This suggested that the pH had reached an unfavourable level, and therefore we examined the effect of the initial pH of the medium.

iv) Effect of Initial pH. The results of cultivating cells of B. brevis 47 for 24 h in medium with an initial pH ranging from 5.5 to 8.5 are shown in Figure 1. It can be seen that as long as the initial pH of the medium is

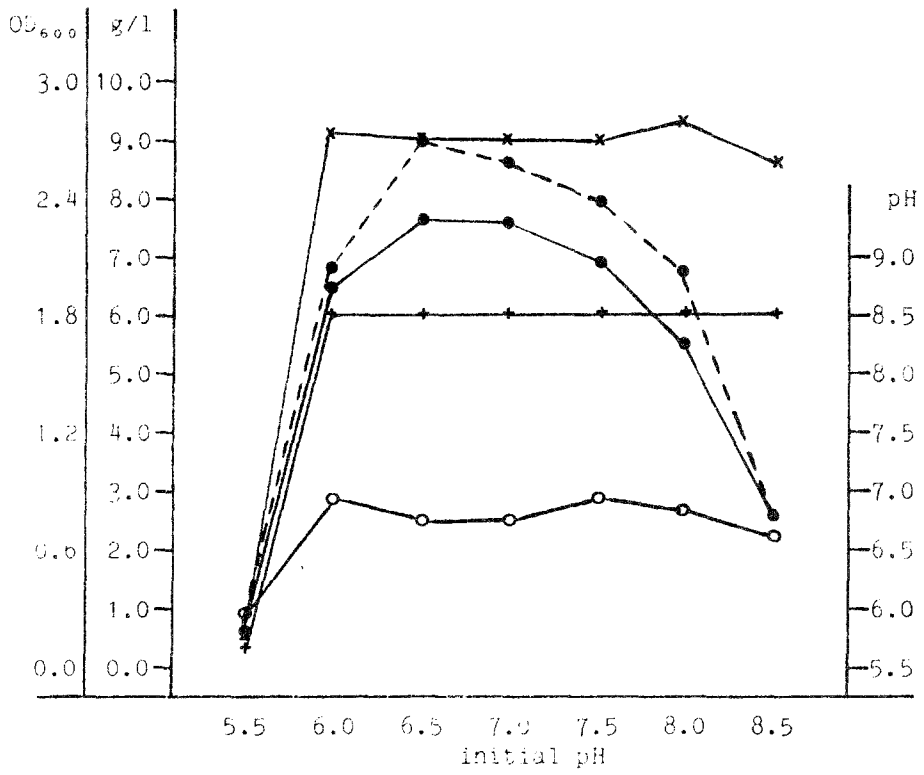

Figure 1: The effect of initial pH on final pH (+), growth (OD_{600})(x), glucose used (g/l)(o), and protein produced (g/l) (secreted ●——●, secreted + cell bound ●- -●) by cells of B. brevis 47 in 24 h.

above 6.0, the cell densities reach the same final values, as do the amounts of glucose consumed and the pH values. However, protein production (extracellular and extracellular + cell bound) appears to be highest when the cells are cultivated in medium with an initial pH of 6.5, and decreases significantly at initial pH values above and below this level. Additionally, the final protein concentration of 9.05 g/l at an initial pH of 6.5 was higher than previously obtained in our work. However, it was interesting to note that glucose consumption was still incomplete.

The above results suggest that the uncontrolled rise of the pH of the medium to a final value of 8.5 may limit protein production. Providing a lower pH at the outset (one above a critical level) may allow the cells to produce additional protein before the final pH of 8.5 is reached. External

regulation of the pH during the course of the fermentation should therefore also enhance protein production.

CONCLUSION

Extracellular protein production by B. brevis 47 is enhanced under conditions of high aeration and relatively large (5% v/v) inoculum sizes. Supplementation of the medium with higher levels of medium components was not found to improve protein production. A slightly lower initial pH did result in a final extracellular protein concentration of 9 g/l. Under the various conditions used, the cells increased the pH of the medium to a final value of 8.5. Greater than 60% of the original amount of glucose also remained unused. Work with fermentors, in which pH and aeration can be readily controlled, is underway in an effort to improve protein production further.

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