Growth Rates and Yields of *Bacillus* Bacteria Can Compare with Those of Yeasts

BACTERIAL CULTURE

Rapid Mass Propagation of Some Bacteria Of the *Bacillus* Genus

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In connection with the development of fermentation processes it has been observed that many bacterial strains of the genus *Bacillus* are able to grow at rates and with cell yields that resemble those for baker's and fodder yeast. In batch propagations with vigorous aeration six identified species and many unidentified soil isolates of bacteria grew on a simple beet molasses medium to give dry cell yields ranging from 20 to 60 grams per 100 grams of sucrose within 6 to 12 hours. Such propagations should offer advantages with respect to continuous operation, minimization of pure-culture precautions, reduction of capital investment, and use of inexpensive media. Attempts to develop such propagations for the production of feed supplements are in progress.

MOST AEROBIC PROPAGATIONS of microorganisms require one or more days between inoculation and harvest of the final propagation stage. This is true, to name a few, for penicillin and the other common antibiotics and for riboflavin and cobalamin (vitamin B_{12}). Primary yeast (bakers' or fodder yeast), on the other hand, requires about 6 to 12 hours for the final stage of a batch process. This type of propagation possesses certain advantages over slower propagations that arise out of the rapid growth rate.

It is the rate of increase in the total cell substance (rather than cell numbers) in the practical range for mass production of cell solids that must be considered. Rapid reproduction rates in very young or very dilute cultures, such as are usually studied by plate counts, were reported long ago for many bacteria.

During the past 8 years the authors have observed that a number of strains of *Bacillus* have mass propagation characteristics resembling those for primary yeast. Thus Stubbs *et al.* in 1947 (*13*) published a growth curve for a strain of *B. subtilis* (ATCC 6633, a subtilin producer) which showed maximum growth with 7 hours. Indeed, the amount of cell substance increased approximately 11-fold in 3 hours. In 1949 a process utilizing *B. megaterium* (NRRL B-938, a cobalamin producer) was announced (*8*). A 14-fold increase in cell substance was reported to occur within 9 hours. Since that time much more rapid propagation rates have been reported by Garibaldi *et al.* (*5*) for this and other strains of *B. megaterium*.

The yield of Schardinger's enzyme by *B. macerans* in aerated oatmeal medium was increased 10-fold over previously reported values, and the propagation period was reduced from several days to 10 to 12 hours by Schwimmer and Garibaldi (*10*).

Laboratory Investigations

During the past 2 years several hundred different stocks of *Bacillus* have been grown in this laboratory in aerated 10-liter batches, in connection with a search for feed supplements (*9*). By far the greater proportion of these have given rapid propagation rates, similar to those mentioned. With 5% of shake flask inoculum, it is customary to observe a lag period of 1 or 2 hours only (by turbidity or packed cell volume) and to obtain maximum yields of cell solids after further aeration for 5 to 7 hours.

The stocks used are mostly unidentified soil isolates, but several dozen named stocks have been grown (Table I). These include *B. licheniformis* (bacitracin

and licheniformin producers), *B. pumilus*, *B. cereus*, and *B. subtilis* (subtilin, bacillin, and rizobacidina producers). However, some stocks have failed to grow rapidly on the first trial, and in general no further attention has been paid to such stocks.

A medium based on beet molasses, ammonium nitrogen, and inorganic salts was used (Table II) for isolation and submerged propagation. Only a few of the strains obtained from culture collections failed to grow luxuriantly on this medium. Indeed, a number of strains grew well on simplified media (*3*, *5*) containing only purified sugar, ammonium nitrogen, and inorganic salts. Ammonium citrate was used for buffering in small scale propagations, but it was omitted and the ammonium phosphate concentration was reduced greatly for large scale runs to which ammonia was added during the propagation.

Propagators (*5*) have been used which achieve vigorous air dispersal and agitation by means of a propeller driven at high speed. In 1- and 10-liter propagators this propeller speed was 1700 r.p.m.; in a 150-liter propagator with a larger propeller, 300 r.p.m. was used. One volume of air was used per volume of culture per minute. On a large scale (4000 gallons) a primary yeast propagator (*2*) has given good results with several strains. It was equipped with a conventional aerator made from a grid of copper pipes with fine holes.

A typical propagation in a primary yeast propagator is shown in Figure 1 for *B. subtilis*. Moist cell volume (determined by centrifuging aliquots) is plotted on a logarithmic scale against time (in hours) on the linear horizontal axis. The rapid nature of the growth and the minor nature of the lag effects induced by transfer are to be noted. Essentially similar propagations have been obtained with other strains of *B. subtilis* (NRRL B-1471, NRRL B-1474, NRS 1074, and subtilin-producing ATCC 6633), with *B. pumilus* (NRRL B-1489), and with the cobalamin-producing *B. megaterium* (NRRL B-938 and others) (*2*, *5*).

It is noteworthy that the final propagation stage involved non-pure-culture equipment. A significant degree of microbial contamination has not been encountered in propagations which have grown with normal rapidity. Bacteriophage was encountered with *B. megaterium* (*5*) and *B. subtilis* (NRS 1074). The phage infections of *B. megaterium* were controlled fairly readily through the selection of resistant strains.

Bacterial cell solid yields with certain members of *Bacillus* approach those obtained with yeast. Such yields may be expressed as percentage of the weight of the sugar supplied. Thus, yields up to 60 grams of dry washed torula yeast are obtained per 100 grams of sucrose; this may be spoken of as a 60% yield, although a theoretical maximum yield cannot be calculated readily. Yields for baker's yeast are usually considered to run between 40 and 50%. Yields of the strains of *Bacillus* under investigation have tended to cluster about the two yields, 25 and 50% (Table I). *B. subtilis* is typical of the first; the cobalamin-producing *B. megaterium* is typical of the second. Of 89 unidentified soil isolates, 58 gave yields in the range 15 to 30% and 15 in the range 45 to 60%. The lower-yielding cultures frequently elaborate large amounts of a polysaccharide, which gives a viscous consistency to the mature culture. This was observed to occur with certain strains, regardless of whether sucrose or glucose was used as the sole carbohydrate.

Table I. Propagation Rates and Cell Solids Yields of Some *Bacillus* Strains

Species	Strain[a]	Hours Required: Lag phase	Hours Required: Max. cell solids	Max. Reproduction Rate[b]	Bacterial Cell Solids Yield, G./100 G. Sucrose
B. cereus	NRS 808	0–1	12	0.34	33
	NRS 992	0–1	14	0.39	43
B. licheniformis	NRS 1082	0–1	7	0.52	25
	NRS 1331	2	12	0.66	23
	ATCC 10,716 (bacitracin)	0–1	9	0.58	26
B. megaterium	NRRL B-938 and others (*4*) (cobalamin)	0–2	7–9	0.9–1.4	~50
B. pumilus	NRRL B-1489	0–1	8	0.68	25
B. simplex	MB-28 (simplexin)		6		~28
B. subtilis	ATCC 6633 (subtilin)	2	9	0.75	
	NRRL B-1467 (subtilin)	0–1	8	0.79	20
	NRRL B-1296 (bacillin)	0–1	6	1.04	26
	NRRL B-1466	0–1	6	1.14	27
	NRRL B-1471	0–1	9	0.85	28
	NRRL B-1473	0–1	7	0.65	27
	NRRL B-1474	0–1	9	0.85	23
	NRRL B-1490	0–1	8	0.91	28
	NRS 1074	0–1	8	0.85	26
	NRS 1143	0–1	12	0.66	22

[a] Identifications refer to American Type Culture Collection, and culture collections of Northern Regional Research Laboratory and of N. R. Smith (*11*). The strain of *B. simplex* was very kindly supplied by H. B. Woodruff, Merck & Co., Inc.

[b] Reproduction rate constant, r, is given by equation: $x_2 = x\ 2^{r(t_2 - t_1)}$ where x_2 and x are amounts of cells at times t_2 and t_1.

Advantages of Process

Some of the advantages may be cited for a process such as has been described. The rapid growth rate suggests that smaller capital investments may be required, due directly to the rapid turnover and to the likelihood that rigorous precautions against contamination may be necessary only for inoculum development.

The same type of economy may also be achieved if, as appears likely, this type of propagation can be adapted readily to a continuous process. Indeed, indirect benefits in the nature of higher yields, more rapid growth, and lower biochemical oxygen demand (BOD) of the fermentation wastes may be expected in addition to the strictly engineering advantages that a continuous process has over a batch process.

To appreciate the possibilities it is necessary to consider that the optimum conditions of aeration, temperature, pH, and nutrient concentrations for a batch process necessarily represent a complex compromise between those that would be optimal for growth and those that would be optimal for elaboration of the desired metabolite, if these functions could be evaluated separately. Requirements for the two functions may, indeed, be antagonistic to each other. In a multiple-stage continuous propagation a segregation of such functions would be possible. Thus the first stage (or tank) might have optimal conditions for growth—for example, high sugar concentration and absence of growth inhibitors. In a second stage, conditions might be optimal for product development—for example, low sugar and the presence of a growth inhibitor such as a toxic precursor or an inhibitor for a cer-

tain enzyme. Recent reports on the advantages of continuous pH control (*4, 7*) and sugar addition (*6, 12*) point in this direction.

Bacillus cells are readily recovered in centrifugal yeast separators, as has been described for *B. megaterium* (*2, 5*). This presents a substantial advantage for recovery cost through elimination of the cost of drying the greater part of the culture. The desired factors must be associated naturally with the cells or be caused to associate with the cells. With *B. megaterium* cobalamin is associated quantitatively with the cells when they are grown in this rapid type of propagation (*5*), but not if they are grown in shake flasks, nor is it true of most other types of cobalamin propagations. Presumably this difference is related to minimal autolysis in the rapid propagation.

The cell substance itself may have incidental protein and vitamin values. These may not be negligible in feed supplements, particularly if several different types of supplements were to be fed at substantial levels.

B. megaterium appears to have potential value as a foodstuff, apart from its cobalamin content. It is more palatable than yeast and it has been shown by Ambrose and DeEds (*1*) not to possess chronic toxicity to rats at concentrations as high as 20% of the ration.

Some members of the *Bacillus* group are thermophilic. The use of such forms might make possible still faster production rates than have been demonstrated with the mesophiles, and certainly would give economies in the cost of the cooling water which is necessary with these rapid propagations. The dangers of microbial and phage contaminations might also be lessened.

The possible low cost for plant, labor, and technical supervision would favor dispersal of plants into consuming areas. This in turn might favor use of local wastes and by-products. Thus it has been reported that waste whey has successfully replaced part of the molasses in the production of *B. megaterium* (*2*). One particularly interesting situation involves tropical agriculture. Animal production in the tropics is relatively undeveloped, in part because of a lack of cheap protein from cereals and legumes. On the other hand, molasses is frequently plentiful and cheap. Perhaps if a small proportion of the molasses were converted to microbial feed supplements that supply vitamins, antibiotics, and proteins, a larger proportion of surplus molasses might be used locally to increase animal production.

Table II. Nutrient Media

	G./Liter		Mg./Liter
Beet molasses (or sucrose)	85 (50)	Mg^{++}	50
		Ca^{++}	20
Diammonium phosphate	8.5	Mn^{++}	50
Diammonium citrate	10	Fe^{+++}	5
Potassium sulfate	2	Zn^{++}	5
pH adjusted to 7.0		Co^{++}	2

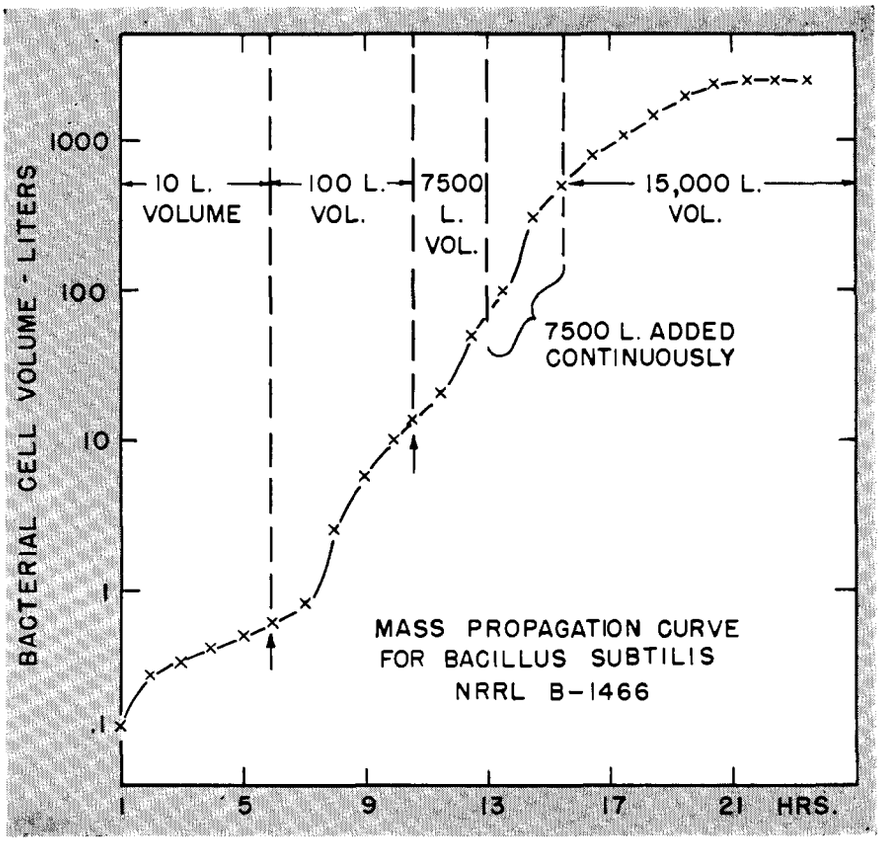

Figure 1. Course of propagation of *B. subtilis* in a primary yeast propagator

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