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Amylase Activity and L-Phenylalanine Overproduction from Starch by an Analog Resistant Mutant of *Bacillus Polymyxa*

Scientific Note

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INTRODUCTION

The potato processing industry generates abundant, underutilized starch wastes (*1*). Bioconversion processes have been developed to utilize these wastes for the production of single cell protein and ethanol (*2,3*). Our efforts are in the direction of developing bioconversion processes for the production of L-phenylalanine and other amino acids from starch wastes.

L-phenylalanine is a key component of the artificial sweetener Aspartame (*4*). Recently it was reported that a *Bacillus polymyxa* mutant BT^R-7, resistant to growth inhibition by β-2-thienylalanine and *p*-fluorophenylalanine, produces L-phenylalanine from starches (*5*). The mutant BT^R-7 is characterized by high amylase activity and alteration in the regulation and synthesis of key shikimate acid pathway enzymes (*5*). Specifically, the mutant has an L-phenylalanine feedback insensitive prephenate dehydratase and increased specific activity of deoxyarabinoheptulosonate-7-phosphate synthase (DAHP synthase). This alteration

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in regulation is also typically seen in other amino acid producing mutants (*6,7*).

This paper outlines an effort to develop kinetic models for growth, amylase activity, and L-phenylalanine production for the mutant BT^R-7. An understanding of these parameters is necessary for design of a large scale process for optimum production of L-phenylalanine. Our preliminary studies also indicate that various starch processing wastes can be used as substrates to produce L-phenylalanine.

MATERIALS AND METHODS

Microorganisms and Media

B. polymyxa ATCC 842 was obtained from American Type Culture Collection (Rockville, MD). BT^R-7 is a mutant derived from *B. polymyxa* ATCC 842 by a two-step ethyl methanesulfonate mutagenesis (*5*). The microorganisms were maintained on starch minimal medium (SMM) agar slants, and stored at 4°C. The composition of SMM used for growth of the parent culture and the mutants (per liter) was as follows: 10.0 g starch, 2.0 g $(NH_4)_2SO_4$, 5.0 g Na_2HPO_4, 3.0 g KH_2PO_4, .2 g $MgSO_4 \cdot 7H_2O$, .2 g NaCl, .05 g $CaCl_2 \cdot 2H_2O$, and 1 mL of trace element solution (*8*). The final pH of the medium was 7.1. Cultures were grown shaken at 37°C and 100 rpm.

Growth Curve, Amylase Activity, and Total Carbohydrate Determinations

B. polymyxa ATCC 842 was incubated shaken at 100 rpm at 37°C in 100 mL of SMM in 250 mL flasks, and samples aseptically removed periodically. Cell growth was measured turbidometrically at 600 nm, and was converted to mg cell dry wt/100 mL, from a calibration curve relating absorbance to cell dry weight. For amylase assays and total carbohydrate determinations, culture broth samples were centrifuged at 10,000 g for 15 min. Extracellular amylase activity in broth samples was determined by following increases in the concentration of reducing sugar (as maltose) over time in pH 6.9 reaction mixtures containing 1 mL samples of culture supernatant incubated with 1 mL of 1% starch, using the 3,5-dinitro salicylic acid method for the detection of reducing sugars (*9*). The μmol of reducing sugar liberated by amylase were calculated from a maltose standard curve. Activities were based upon initial linear reaction rates, and 1 U of amylase activity was defined as the amount of enzyme liberating 1 μmol of reducing sugar (as maltose)/min at 25°C and pH 6.9. For determination of total carbohydrate, 1 mL samples of culture supernatant were assayed by the anthrone method (*10*). In this assay, carbohydrate depletion was estimated by measuring changes in absorbance at 540 nm, based on a standard curve prepared with starch. Total carbohydrate in culture supernatnat samples was expressed as grams of carbohydrate/100 mL of medium.

High Pressure Liquid Chromatography (HPLC)

Shake flask supernatant samples were taken every 12 h through 96 h. The samples were mixed with methanol (1:1) and filtered through a 45 μm filter (Gelman Sciences, Inc., Ann Arbor, MI). Then, 50 μL of filtrate was injected into a Hewlett-Packard (Bellevue, WA) 1090A HPLC equipped with a HP-1040A diode array detector and a 5 μL sample-applying loop. The mobile phase consisted of a gradient of acidic water, pH 3.25 (H_2SO_4), and methanol at an initial concentration of 20% methanol. The methanol concentration was held at 20% for 2 min, increased to 60% over the next 2 min, and then held at 60% for 2 min, after which it was returned to 20% over the last 2 min (total run time, 8 min). A microbore reverse-phase column (100 by 2.1 mm inside diameter; Hewlett Packard) of Hypersil ODS with a 5 μm particle diameter was used with a flow rate of .4 mL/min and a column temperature of 40°C. During each run, a chromatogram was recorded at 258 nm, and the UV absorbance spectrum (250–350 nm) of each chromatographic peak was recorded at its front, apex, and backside. Under these conditions, the retention time of L-phenylalanine was 1.35 min, and it exhibited a sharp absorption maximum at 258 nm. Retention times varied slightly. This was corrected by periodic chromatography of L-phenylalanine standards and by examination of UV absorption spectra. A standard curve for L-phenylalanine was prepared by plotting a known concentration of L-phenylalanine vs peak area units, recorded with a Hewlett-Packard 3390 integrator.

Protein Determinations

Protein concentration was determined by the method of Lowry et al. (*11*), using bovine serum albumin as the standard.

Chemicals

L-phenylalanine, 3,5-dinitro salicylic acid, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO), HPLC-grade methanol and anthrone from Baker Chemical Co. (Phillipsburg, NJ), and soluble starch from Difco Chemical Co. (Detroit, MI). All other chemicals were laboratory reagent grade.

RESULTS AND DISCUSSION

Growth Kinetics and Carbohydrate Utilization

The growth curves in SMM were similar for both the parent and the mutant BT^R-7 (Figs. 1 and 2). Exponential growth was observed between 0–32 h, with little lag phase. This was followed by a declining growth

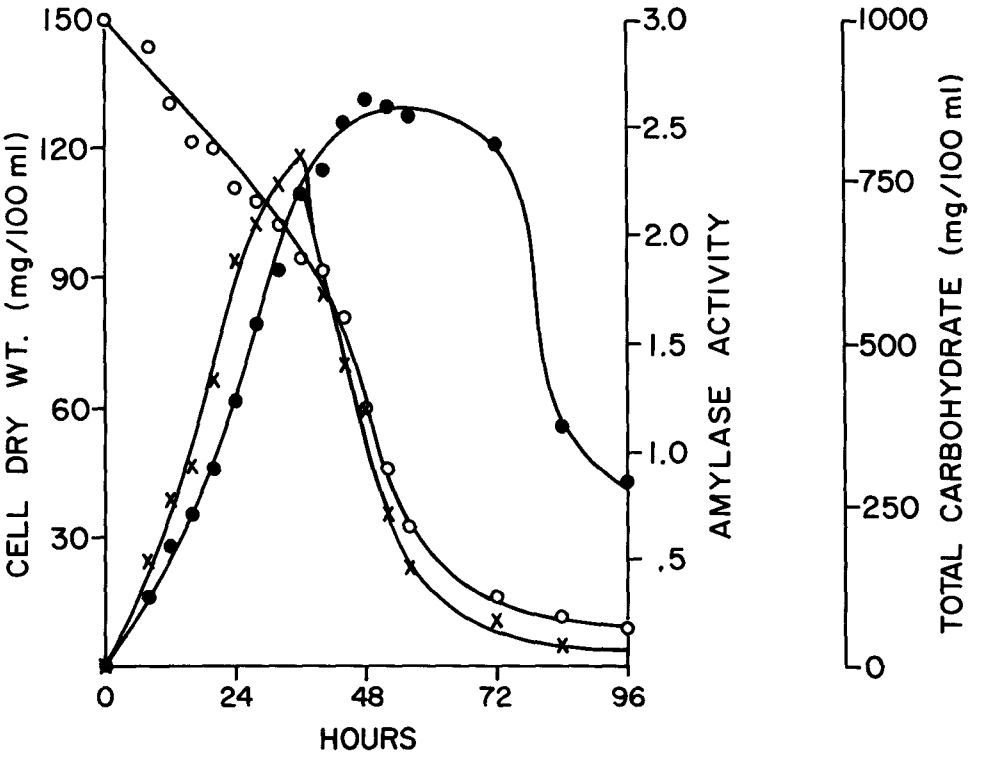

Fig. 1. Growth, amylase activity, and carbohydrate utilization pattern of *Bacillus polymyxa*; parent ATCC 842 incubated shaking at 37°C for 4 d in starch minimal medium. Growth (cell dry wt in mg/100 mL of SMM) (●); amylase activity expressed as μmol reducing sugar/min/mL of samples (X); and carbohydrate utilization (O).

phase between 32–48 h. Stationary phase and death phase occurred between 48–84 h. Specific growth rate (μ_{max}) was calculated using

$$dX/dt = \mu_{max}X \quad (1)$$

for the exponential phase where X is the cell dry weight (mg/100 mL). The μ_{max} values for the parent and BT^R-7 were .053 h^{-1} and .051 h^{-1}, respectively, indicating similar specific growth rates. However, carbohydrate utilization was lower in the parent than the mutant BT^R-7 during the logarithmic growth phase. At the end of the log phase the parent culture had utilized only 60% of the total carbohydrate, compared to 80% utilization by BT^R-7. This difference in carbohydrate utilization was reflected in Y_{sx} (yield coefficient; *see* Eq. [4] and Fig. 3). The difference in Y_{sx} can be explained by characteristics of the mutant BT^R-7, which has a deregulated shikimic acid pathway (5) that increased the net flux of carbon into the L-phenylalanine pathway. This diversion of carbon to product formation could result in reduced carbon for biomass. However, results show that biomass production was constant for both parent and

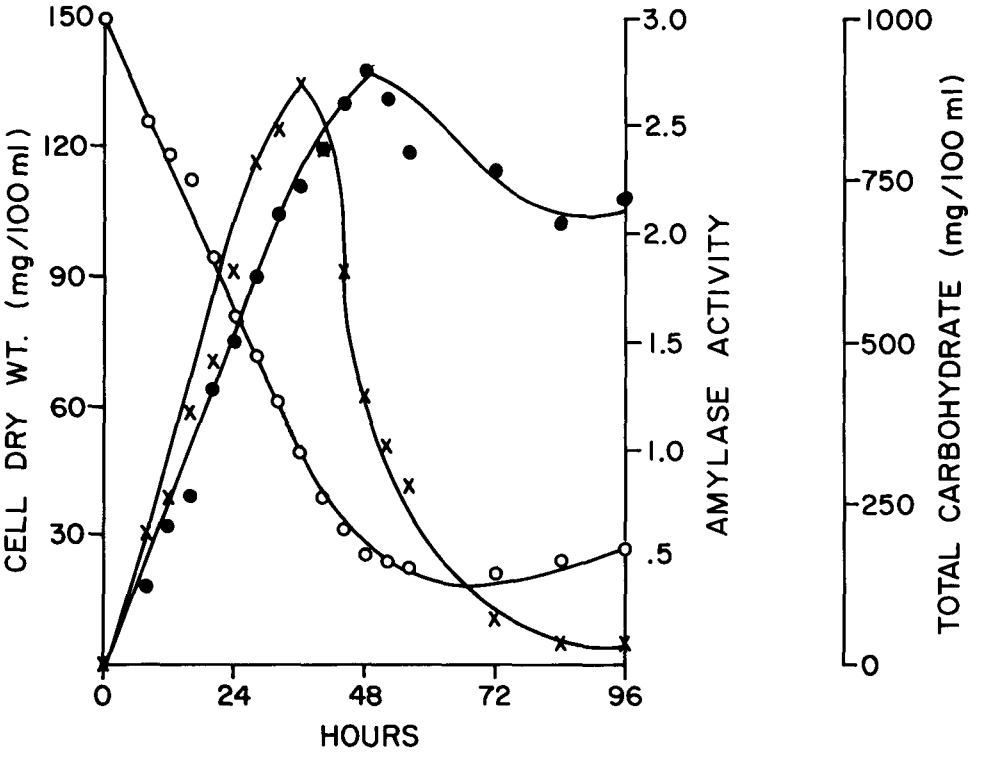

Fig. 2. Growth, amylase activity, and carbohydrate utilization pattern of *Bacillus polymyxa*; mutant BT^R-7 incubated shaking at 37°C for 4 d in starch minimal medium. Growth (cell dry wt in mg/100 mL of SMM) (●); amylase activity expressed as micromole reducing sugar/min/mL of sample (X); and carbohydrate utilization (○).

mutant. Thus, the extra carbon flow for L-phenylalanine must come from enhanced starch utilization by BT^R-7 (Figs. 1 and 2).

Amylase Activity

Amylase activity increased similarly in the parent and the mutant BT^R-7 over time (Figs. 1 and 2). The increase was growth associated until 36 h, the period of maximum activity. Between 36–96 h there was a constant decay in the amylase activity. The specific amylase activity (E) was calculated (considering the exponential phase only) using the equation

$$dE/dt = \mu^E_{max} E \tag{2}$$

μ^E_{max} was .055 h^{-1} and .053 h^{-1}, respectively, for the parent and the mutant BT^R-7, indicating similar amylase synthesis rates.

The decay for the period 36–96 h showed an exponential loss of activity. The decay constant k was calculated using the equation

$$dE/dt = -k\,E \tag{3}$$

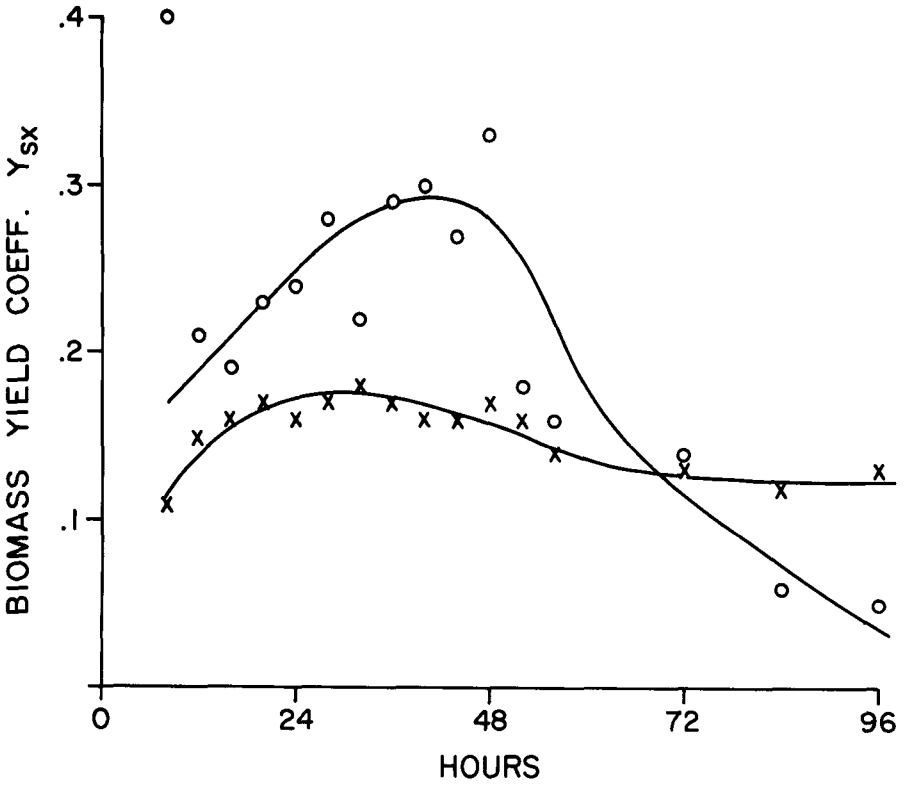

Fig. 3. Yield coefficient; Y_{sx} (mg cell dry wt/mg of carbohydrate utilized) for parent ATCC 842 (O); and mutant BT^R-7 (X).

Values of k for the parent and BT^R-7 were .063 h^{-1}, and .075 h^{-1}, respectively, showing no statistically significant difference as determined by Fisher LSD for $p > .05$. The growth associated pattern of amylase activity may be important for assuring adequate availability of monosaccharides from starch. The exponential decay of amylase activity could affect the complete utilization of all the available starch, which could be the result of extracellular proteolytic activity. Extracellular proteolytic activity is a common feature of the genus *Bacillus* (12).

Yield Coefficients

The yield coefficient Y_{sx} is a measure of the cell dry weight accumulated (dX) per weight of carbohydrate consumed (dS).

$$-dS/dt = 1/Y_{sx}\, dX/dt \quad (4)$$

As seen from Fig. 3, Y_{sx} is consistently lower in the mutant BT^R-7 than in the parent. BT^R-7 has a deregulated L-phenylalanine biosynthetic pathway that results in increased carbon flow while maintaining the same cell growth as the parent strain. Thus the mutant utilizes carbohydrate at a higher rate to meet the needs of increased flow carbon for

L-phenylalanine overproduction, and this was reflected in lower Y_{sx} values (Fig. 3).

To further enhance the efficiency of L-phenylalanine production, it may be necessary to maintain the rate of biomass production while improving the efficiency of utilization. The key to this may be higher amylase activity and reduced proteolysis. Pretreatment of starch wastes by amylases may also be a feasible approach.

The yield coefficients Y_{xE} and Y_{sE} refer to the yield of amylase activity per unit of biomass produced and substrate consumed, respectively.

$$dX/dt = 1/Y_{xE}\, dE/dt \tag{5}$$

and

$$-ds/dt = 1/Y_{sE}\, dE/dt \tag{6}$$

Values of Y_{sE} and Y_{xE} (Table 1) indicate little correlation in terms of L-phenylalanine production and cell physiology. However, these yield coefficients are good indicators of changes in amylase synthesis.

L-Phenylalanine Production

Further attempts are being made in our laboratory to better understand starch utilization physiology in *Bacillus polymyxa* for production of variety of amino acids from starch processing wastes. The mutant BT^R-7

Table 1
Yields of Amylase Activity: Y_{XE}, U/mg Dry Biomass, and Y_{SE}, U/mg Carbohydrate Consumed, for *Bacillus polymyxa* ATCC 842 and Mutant BT^R-7 During Growth in SMM medium

	Y_{XE}		Y_{SE}	
Time, h	Parent	BT^R-7	Parent	BT^R-7
8	.030	.030	.01	.004
12	.030	.020	.006	.004
16	.030	.030	.005	.005
20	.030	.020	.007	.004
24	.030	.020	.007	.003
28	.025	.030	.007	.004
32	.020	.020	.007	.004
36	.020	.020	.006	.004
40	.015	.020	.004	.003
44	.010	.010	.003	.002
48	.010	.009	.002	.002
52	.006	.008	.001	.001
56	.004	.007	.0006	.001
72	.002	.002	.0002	.0003
84	.002	.001	.0001	.0001
96	ND	.001	ND	.0001

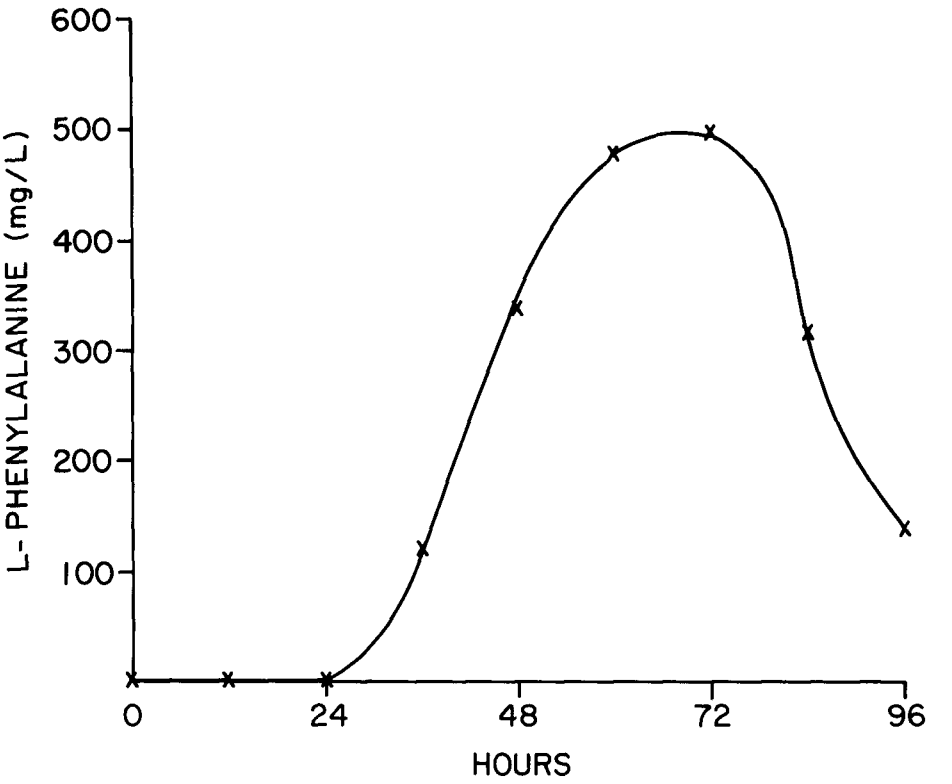

Fig. 4. L-Phenylalanine accumulation (mg/L from 1% starch) with *Bacillus polymyxa;* mutant BT^R-7.

produced .5 g/L and .15 g/L of L-phenylalanine and L-tyrosine, respectively, when grown in SMM. The maximum L-phenylalanine concentration was at 72 h, in the stationary phase (Fig. 4). This late production could be the result of the low transport rate of L-phenylalanine from cells during the exponential phase. L-phenylalanine concentrations increase in the stationary phase as a result of cell lysis. Studies are underway to monitor the internal L-phenylalanine level to determine transport effects for L-phenylalanine.

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