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Sucrose Catabolism in *Clostridium pasteurianum* and Its Relation to N_2 Fixation

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The growth constant and Y (sucrose) (grams of cells per mole of sucrose) for NH_3-grown cultures of *Clostridium pasteurianum* were 1.7 times those of N_2-grown cultures, whereas the rate of sucrose utilized per gram of cells per hour was similar for both conditions. The Y (sucrose) of chemostat cultures grown on limiting NH_3 under argon at generation times equal to those of N_2-fixing cultures was less than that of cultures grown on excess NH_3, but cells of NH_3-limited cultures contained the N_2-fixing system in high concentration. The concentration of the N_2-fixing system in whole cells, when measured with adenosine triphosphate (ATP) nonlimiting, was more than twofold greater than the amount needed for the N_2 actually fixed. Thus, energy production from sucrose, and not the concentration of the N_2-fixing system nor the maximal rate at which N_2 could be fixed, was the limiting factor for growth of N_2-fixing cells. Either NH_3 or some product of NH_3 metabolism partially regulated the rate of sucrose metabolism since, when cultures fixing N_2, growing on NH_3, or growing on limiting NH_3 in the absence of N_2 were deprived of their nitrogen source, the rate of sucrose catabplism decreased. Calculations showed that the rate of ATP production was the growth rate-limiting factor in cells grown on N_2, and that the increased sucrose requirement of N_2-fixing cultures in part reflected the energy demand of N_2 fixation. Calculations indicated that whole cells require about 20 moles of ATP for the fixation of 1 mole of N_2 to 2 moles of NH_3.

N_2-fixation in cell-free extracts of *Clostridium pasteurianum* is an energy-requiring process. It has been reported that two adenosine triphosphate (ATP) molecules are consumed per electron transferred in *C. pasteurianum* (9) and five ATP molecules are consumed per electron pair in *Azotobacter vinelandii* (5). N_2-fixing cultures should have a significantly higher requirement for energy than do cultures growing on NH_3, and the anaerobe *C. pasteurianum* may require more than two sucrose molecules for each molecule of N_2 fixed.

In this paper, the difference in sucrose utilization between N_2-fixing and nonfixing cultures is quantitated, and the regulation of catabolic activity is studied in relation to nitrogen metabolism.

MATERIALS AND METHODS

The medium used for the continuous cultures was 2×10^{-4} M in $MgSO_4$ and $FeCl_3$, 2×10^{-5} M in $MnSO_4$ and $CaCl_2$, 2×10^{-6} M in $ZnSO_4$, $CuSO_4$, and $CoCl_2$, and 0.15 M in potassium phosphate, *p*H 6.9. Except for those experiments on the effects of sucrose limitation, the medium was made 1% in sucrose; 0.1 mg of biotin was added before autoclaving. Eight liters of this medium was autoclaved in 9-liter serum bottles. Since the medium contained some insoluble salts, it was stirred throughout the experiments. A 500-ml vacuum flask was used as the growth vessel; media were introduced through glass tubing fitted in a rubber stopper and connected to a tygon tubing inlet from the reservoir. The flow of medium through the tygon tubing was regulated by use of a Sigmamotor adjustable pump. The side arm of the growth vessel provided the exit for the effluent culture. Argon or N_2 was introduced through a sintered-glass sparger fitted through the rubber stopper of the flask; the gas flow was monitored with a flow meter and kept below 200 ml/min. The culture was kept in suspension by magnetic stirring. The growth vessel was kept in a constant temperature water bath at 30 C. Ammonium sulfate was used as the NH_3 source in all experiments.

The effluent medium of the continuous cultures could not be chemically assayed for N_2 content; thus, to show that N_2 was not a limiting nutrient in these experiments, growth was used as an index. Since the N_2-fixing cultures contained sucrose in excess, the cells were allowed to increase in concentration by stopping the flow of the medium until the density was twice that of the experimental cell concentration but with no change in the flow rate of N_2 through the growth flask. The flow of the medium was resumed and, if the culture equilibrated at the higher population density with the same growth constant it had at the lower density, it was assumed that at the lower density the external supply of N_2 (as well as other components in the medium) was nonlimiting.

In the experiments with ^{14}C-labeled sucrose, the growth medium was the same as just described, except that the phosphate concentration was 6.6×10^{-3} M, the $CaCl_2$ was omitted, and 3 g of $CaCO_3$ per liter was used to buffer the system. Initial concentration of sucrose varied from 27 to 35 mM for the three experiments performed. Total ^{14}C as uniformly labeled sucrose varied from 10^6 to 10^7 counts/min per growth flask. Cultures (100 ml) were grown in 500-ml aspirator bottles, the tubulation of which was fitted with heavy-walled rubber tubing closed with a screw clamp from which samples were withdrawn. The cultures were grown under flowing N_2 for at least three generations in the presence of the labeled sucrose, and the experiment was terminated by centrifugation of the cultures at 500 × *g*. The cells were washed with cold 1% sucrose in 0.1 M phosphate (*p*H 7.0), centrifuged, suspended in a small volume of distilled water, and disrupted by sonic treatment. Appropriate amounts of the solubilized cells and the supernatant medium were placed in Bray's solution (4) and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Anthrone reagent was used for all sucrose analyses (1). Determination of the rate of sucrose used per gram of cells in the chemostat was accomplished by methods previously established (7). Y (sucrose) (grams of cells produced/mole of sucrose) and Y (ATP) (grams of cells produced/mole of ATP) were determined as suggested by Beauchop and Elsden (3). Ammonia was determined by the microdiffusion technique of Conway (6) and nitrogen was determined by Kjeldahl digestion (2). To determine the nitrogen content of NH_3-grown cells in the continuous culture, the amount (μmoles) of NH_3 per ml of the reservoir and effluent mediums was measured, the difference was multiplied by the volume of the culture, and the result was divided by the amount (grams) of cells in the culture. The result from 14 independent determinations was 7.5 ± 1.1 mmole of NH_3 per g of dry cells or 105 mg of nitrogen per g of cells. For batch cultures, the differences in NH_3 content of samples taken at different times was divided by the corresponding change in mass of the cells. No significant difference between continuous and batch cultures was observed, and no significant differences were found between cultures grown under an atmosphere of argon or N_2. Cells from chemostat cultures grown on limiting NH_3 had the same nitrogen content per gram (dry weight) as did nonlimited cultures.

The nitrogen content of N_2-fixing cells determined by Kjeldahl digestion averaged (four determinations) 6.6 ± 0.5 mmoles per g of dry cells or 92 mg of nitrogen per g of cells.

Acetic and butyric acids in cultures, culture filtrates, and standard solutions were measured by gas chromatography with a Varian Aerograph (model 600-B) with a free fatty acid phase (FFAP) column supplied by the Wilkens Co.

Estimation of N_2 fixed by measuring acetylene reduction was based on a modification (10) of the method of Koch and Evans (8).

Optical density (OD) measurements of cultures were made at 660 mμ in 1-cm cuvettes; a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.) was used. The samples were diluted in 4% acetic acid (to dissolve basic salts) so that readings between 0.10 and 0.31 were always obtained. One liter of culture at an OD of 1.00 contained 380 mg of cells (dry weight).

RESULTS

Sucrose utilization by cells grown on N_2 and on NH_3. Sucrose utilization was studied under three different conditions: (i) optimally growing N_2-fixing cultures, with sucrose present at all times in the effluent medium; (ii) optimally growing NH_3-supplemented cultures grown under an atmosphere of argon, with both NH_3 and sucrose present in the effluent medium; and (iii) NH_3-limited cultures grown under argon, with sucrose but without NH_3 present in the effluent medium and with the growth rate of the culture regulated by the rate of supply of NH_3 to equal that of the N_2-fixing culture. The results of these experiments, in Table 1, show that the rate of sucrose utilization per gram of cells was similar under all three conditions, whereas the Y (sucrose) and the growth constant for NH_3-grown cultures were 1.7 times those of N_2-fixing cultures. Under the third condition, the Y (sucrose) was similar to that of a N_2-fixing culture. In addition, under the third condition, the bacteria apparently consumed up to 40% of the sucrose in reactions other than those of normal biosynthesis, since, because of nitrogen limitation, only 60% of the total sucrose consumed would be required to produce the same amount of NH_3-grown cells under NH_3-sufficient conditions. This could be taken as evidence that the cell's catabolic activity is not regulated to its biosynthetic needs. Further investigations, however, have shown that cultures grown on limiting NH_3 in the absence of N_2 possess a highly active N_2-fixing system and behave more like "N_2-fixing" cultures (*in preparation*). ATP consumed by the nonfunctioning nitrogenase (5, 9) could be responsible for at least part of the excess sucrose utilization.

The above results were reexamined with radioactive sucrose to determine how much carbon of the sucrose fermented was incorporated into the cells under the two conditions. When paired cultures, one growing on N_2 and one growing on NH_3, were grown in the presence of uniformly labeled ^{14}C-sucrose, the percentage carbon incorporated into cellular material averaged 9.8 for

TABLE 1. *Rate of sucrose utilization and cell yield of Clostridium pasteurianum grown in a chemostat on excess sucrose under N_2-fixing and nonfixing conditions*[a]

Nitrogen source	Growth constant	Y (sucrose) (g of cells/mole of sucrose)	Amt (mmoles) of sucrose used per g of cells per hr
N_2 (excess)	0.40	36.6	10.9
NH_3 (excess)	0.69	63.0	10.9
NH_3 (limited)[b]	0.41	38.0	10.6

[a] The figures given for the N_2-fixing state are averages of six independent determinations; those for the optimally growing NH_3-dependent state are averages of two independent determinations; and those for the NH_3-limited state are averages of three independent determinations.

[b] Chemostat with cells growing on NH_3-limited medium so that the doubling time is the same as in the N^2-fixing continuous culture. Gas phase was argon.

TABLE 2. *Cell yield and rates of sucrose utilization for sucrose-limited cultures under N_2-fixing and nonfixing conditions*[a]

Nitrogen source	Concn of sucrose in the reservoir	Growth constant	Y (sucrose) (g of cells/mole of sucrose)	Amt (mmoles) of sucrose used per g of cells per hr
	mM			
N_2[b]	5.95	0.385	43.2	8.95
	5.95	0.217	39.4	5.34
	11.2	0.365	46.2	7.70
	11.2	0.389	43.4	8.68
	11.2	0.217	46.2	4.55
NH_3 (excess)	5.95	0.568	73.1	7.58
	5.95	0.217	70.2	2.76
	11.2	0.555	76.0	6.50
	11.2	0.389	69.5	6.00

[a] All measurements were made in a chemostat culture originally derived from a N_2-fixing inoculum of *C. pasteurianum*. Growth constants were changed by adjusting the flow rate of medium into the chemostat. All cultures were limited in sucrose but to varying degrees.

[b] The maximal possible rate of N_2 fixation by N_2-fixing cells was estimated by acetylene reduction and found to be two or more times that required to maintain the maximal growth constant.

the N_2-fixing culture and 17.1 for the NH_3-grown culture. In an experiment with N_2-fixing cells, of a total of 1.58×10^6 counts/min of the sucrose-*U*-^{14}C used, 1.56×10^5 counts/min was found in the cells, whereas with NH_3-grown cells, of a total of 1.24×10^6 counts/min of sucrose-*U*-^{14}C used, 2.12×10^5 counts/min was found in the cells. The remainder was accounted for in CO_2 and butyric and acetic acids. Since 9.8% and 17.1% sucrose carbon incorporated correspond to Y sucrose values of approximately 31 and 55, respectively (assuming the cells are 45% carbon), these results are in reasonable agreement with those of Table 1.

Two hypotheses are consistent with the results in Table 1. (i) Catabolism is not a regulated process and the rate of N_2-fixation is the growth rate-limiting factor in N_2-fixing cultures; and (ii) the rate of energy supply is the growth rate-limiting factor in both conditions and is simply at its maximum in both states. Senez (11) gave the former interpretation to results obtained with *Desulfovibrio desulfuricans*. To test the first hypothesis with *C. pasteurianum*, a situation was created in which energy supply was clearly the factor limiting growth. By regulating the flow rate (limiting sucrose), two N_2-fixing growth conditions were compared, one in which the bacteria had a growth rate just slightly less than maximum and one in which the growth rate was decreased to 50% of that of the nonlimited culture. Y (sucrose) results obtained were compared with those of similarly regulated cultures on media supplemented with excess NH_3 (Table 2). Since (i) there was no significant increase in efficiency of sucrose utilization [Y (sucrose)] when the maximal possible rate of N_2 fixation was clearly not the growth rate-limiting factor and (ii) the addition of NH_3 increased the Y (sucrose) at least part of the increased sucrose requirement of N_2-fixing cultures reflected the ATP demand for N_2 reduction.

In addition, it was shown that if ATP available to N_2 fixation was made less limiting by measuring N_2 fixation in nongrowing cultures, such cultures could fix over twice the amount of N_2 needed to maintain their normal rate of growth (*in preparation*).

When the growth rates of N_2-fixing and NH_3-grown cultures were controlled by sucrose limitation, a plot of mmoles sucrose used per gram of cells per hr against growth rate (Fig. 1) showed that the ratio of the rates (N_2-fixing to NH_3-grown) increased from 1.5 at a growth rate of 0.40 to 2.8 at a growth rate of zero. At the extrapolated zero growth rate, the N_2-fixing and NH_3-grown cultures consumed sucrose at rates of approximately 1.4 and 0.5 mmoles per g of cells per hr, respectively. Whether the sucrose consumed at zero growth rate is a result of uncoupled ATP hydrolysis or use in "maintenance" is unknown. The difference between the two conditions of 0.9 mmoles per g of cells per hr could be a result of

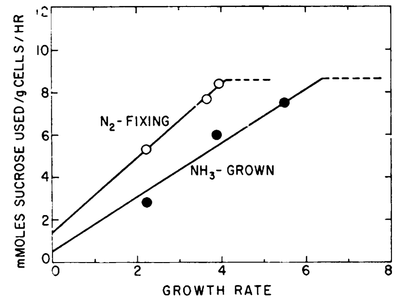

FIG. 1. *Rate of sucrose used as a function of growth rate for N_2-fixing and NH_3-grown cultures. For conditions, see Table 2 and Materials and Methods.*

TABLE 3. *End products of sucrose fermentation by Clostridium pasteurianum*

Nitrogen source	*p*H of medium	A/S (μmoles/ μmoles)	B/S (μmoles/ μmoles)	A/B	Carbon recovered as A and B[b]
					%
N_2	4.6	1.18	1.36	0.90	64
	4.7	1.12	1.30	0.86	62
	6.1	1.34	1.39	0.95	69
	6.4	1.24	1.37	0.91	66
NH_3	4.6	1.29	1.54	0.84	73
	6.5	1.34	1.44	0.93	70
	6.7	1.11	1.38	0.87	64

[a] A (acetate), B (butyrate), S (sucrose).

[b] The remainder of the carbon is in the CO_2 evolved and the cells produced.

ATP consumed by the N_2-fixing system producing H_2 in the absence of N_2.

Energy yield from sucrose by C. pasteurianum. Before meaningful comparisons between sucrose utilization by N_2-fixing and NH_3-grown cells could be made, it was necessary to establish that the ratio of ATP to sucrose used was the same in both cell types. Since the enzymes for typical glycolysis are present in extracts of *C. pasteurianum*, the principal pathway of hexose catabolism and energy production was assumed to be the Embden-Meyerhoff-Parnas pathway, and at this stage the energy yields would be similar. Further metabolism of pyruvate involves two additional energy-yielding steps involving ATP:acetate phosphotransferase and ATP:butyrate phosphotransferase. Since butyric and acetic acids are the fermentation products of these energy-yielding steps, any change in their ratio to each other and to the sucrose consumed would be an index of any differences in the energy yield (ATP) from sucrose. The results of several experiments (Table 3) show that, within experimental error, these ratios were the same under all conditions.

Does sucrose catabolism decrease during N_2 starvation? The question of whether sucrose catabolism is a regulated process was investigated since the answer would have direct bearing on whether N_2 fixation is the only other energy-consuming process after biosynthesis. A N_2-fixing continuous culture was maintained at a steady state (OD of 1.15 for 12 hr) and the rate of sucrose utilization was measured; the flow of medium through the growth vessel was stopped and the gas phase was changed to argon. Measurement of OD and sucrose at 15-min intervals over 2 hr showed that the rate of sucrose catabolism decreased to less than 25% of the control rate (Fig. 2). To show that this was not a result of cell death during the period of nitrogen deprivation, an N_2 atmosphere was restored at the end of 2 hr and sucrose metabolism and growth returned to their former rates.

The same experiment was performed with a culture equilibrated under an atmosphere of argon with the NH_3 concentration of the medium such that less than 1 μmole/ml remained in the effluent. At the beginning of the nitrogen deprivation period, the reservoir was changed to NH_3-free medium and the flow was stopped. The results in Fig. 3 show that, after the residual NH_3 was consumed, the OD remained constant and the rate of sucrose catabolism decreased to less than 25% of the control rate.

If the low Y (sucrose) observed with cultures grown on limiting NH_3 (Table 1) resulted from ATP utilization by a nonfunctioning N_2-fixing enzyme system, then it might be expected that N_2-fixing cells and NH_3-limited cells (cells possessing the N_2-fixing system but growing on limiting NH_3 in the absence of N_2) would consume more sucrose in a period of nitrogen deprivation than nitrogenase-free cells (cells grown on excess NH_3). A comparison of sucrose consumption between growing and nongrowing cultures of N_2-fixing, NH_3-grown, and NH_3-limited cells showed that under all conditions sucrose consumption was depressed by nitrogen starvation. With sucrose in *excess*, sucrose catabolism in N_2-fixing and NH_3-grown cultures averaged about 13.6 and 12.6 mmoles per g of cells per hr under the nitrogen-sufficient conditions, and 6.3 and 4.3 mmoles per g of cells per hr when no nitrogen was present. These results, which are higher than those of Fig. 1 at zero growth rate with sucrose as the limiting substrate, suggested that, when sucrose is in excess and nitrogen is limiting, more uncoupled ATP hydrolysis occurs. The results with several cultures grown on limiting NH_3

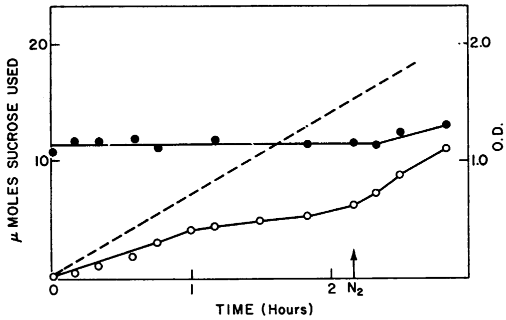

FIG. 2. *Rate of sucrose utilization by a N_2-fixing culture during nitrogen starvation. Symbols:* ○, *μmoles of sucrose used per ml;* ●, *optical density; and dotted line, μmoles of sucrose used per ml by a growing culture.*

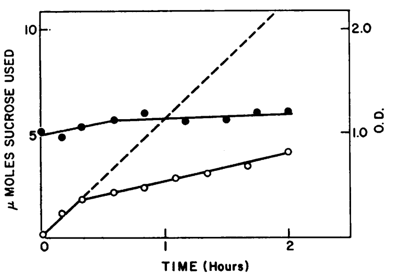

FIG. 3. *Rate of sucrose utilization by an NH_3-grown culture during nitrogen starvation. Symbols:* ○, *μmoles of sucrose used per ml;* ●, *optical density; and dotted line, μmoles of sucrose used per ml by a growing culture.*

showed a decrease in sucrose catabolized, but the decrease was variable, possibly because of the different composition of the N_2-fixing system. This is now under investigation.

DISCUSSION

Since each molecule of sucrose catabolized by *C. pasteurianum* yields four molecules of pyruvate and four molecules of ATP via the Embden-Meyerhoff-Parnas pathway, and the four pyruvate molecules in turn are catabolized to yield 1.26 molecules of acetate, 1.26 molecules of ATP, and 1.38 molecules of butyrate, the minimal gain of ATP per sucrose molecule would be 5.26 molecules. If an ATP molecule is also produced via phosphotransbutyrylase for each molecule of butyrate formed from butyryl-S-coenzyme A (12), the energy yield would be 6.64 ATP molecules per sucrose molecule. Finally, if the utilization of sucrose is initiated by phosphorolysis instead of by hydrolysis, the maximal yield of ATP per sucrose molecule would be 7.64 (unless there are other unknown sources of ATP). Since the maximal Y (sucrose) for NH_3-grown cultures is approximately 79, if the yield of ATP is 7.64/sucrose, then the Y (ATP) (3) would be 10.3. Similarly, since the maximal Y (sucrose) for N_2-fixing cultures is 48, the Y (ATP) would be 6.3. If the energy demands for biosynthesis and assimilation of common material in both N_2-fixing and NH_3-grown cultures are the same and if the efficiency of ATP utilization were not decreased because of the slower growth rate imposed by nitrogen limitation, the ATP available for N_2-fixation would be 65 mmoles per g of cells. Since 1 g of cells contains 6.6 mmoles of nitrogen which corresponds to 3.3 mmoles of N_2 fixed, the ATP to N_2 ratio would be about 20. When compared to the best efficiency found in cell-free extracts (12 ATP per N_2 fixed), this ratio suggested that some energy is lost as a result of the slower growth rate. If this is corrected for the difference in the rate of sucrose used at zero growth rate (Fig. 1), the ratio would be about 13 ATP per N_2.

Since the maximal growth rate constant for N_2-fixing cells is 0.40 and the nitrogen content of these cells is 6.6 mmoles per g of cells, the maximal rate of N_2 fixation would be 1.32 mmoles of N_2 fixed per g of dry cells per hr ($1.32 \div 3.3 = 0.40$). This would correspond to a requirement of 15.9 mmoles of ATP per g of dry cells per hr if 12 ATP are required per N_2 fixed (10) or of 26.4 mmoles of ATP per g of dry cells per hr if 20 ATP are used per N_2 fixed. The average minimal amount (mmoles) of sucrose used per gram of cells per hour (8.5; Fig. 1) with sucrose limiting is equivalent to 65 mmoles of ATP per gram of dry cells per hr. Based on the ATP needed for the production of NH_3-grown cells, up to 40% of 65 mmoles of ATP per g of dry cells per hr, or 26.0 mmoles of ATP per g of dry cells per hr, is not needed for cellular biosynthesis. This agrees with the value of 26.4 based on 20 ATP molecules used per N_2 molecule fixed. Since the N_2 fixed based on 12 ATP per N_2 would require a rate of 15.9 mmoles of ATP per g of dry cells per hr, 10.1 mmoles of ATP per g of dry cells per hr either was used for "maintenance" or was lost through hydrolysis, possibly via the N_2-fixing system.

The minimal rate of N_2 fixation estimated by measuring acetylene reduction by intact cells of *C. pasteurianum* (*in preparation*) is 58 μmoles of N_2 fixed per g of dry weight per min or 3.5 mmoles of N_2 fixed per g of dry weight per hr. If this were the factor limiting the growth rate of the culture, the growth rate constant would be 1.06. Since the growth rate of the N_2-fixing culture is 0.40, this is not the limiting factor and over a twofold excess of enzyme is present (1.06/0.40 or 3.5 mmoles of N_2 fixed per g of dry weight per hr $\div$ 1.32 mmoles N_2 fixed per g of dry cells per hr). In measuring the highest rate at which N_2 could be fixed, we assumed, because only acetylene and argon were present in the reaction vessel, that the cells were not growing and that the energy supply to the N_2-fixing system was nonlimiting. If it were limiting, the maximal rate of N_2 fixation would be even greater.

One possible mechanism through which ATP could be wasted by cells containing the N_2-fixing system could be through ATP utilization and H_2 production by an ineffecient N_2-fixing system (5). N_2 fixation by purified nitrogenase components of this organism has always been accompanied by H_2 evolution; in fact, between 20 and 50% of the ATP source supplied in the presence of N_2 can be consumed through this "ancillary" function of the N_2-fixing system. The results reported here suggest that about 10% of the ATP may be consumed by this mechanism.

When sucrose and the nitrogen source are in excess, NH_3-grown and N_2-fixing cells have similar rates of sucrose catabolism. Of the ATP available from this sucrose catabolism, NH_3-grown cells appear to use 94% in biosynthesis and the remainder (6%) either is used in other functions or is lost to the cell through uncoupled ATP hydrolysis. N_2-fixing cells under the same conditions appear to use 55% of the energy in biosynthesis, 6% in the same unknown functions as NH_3-grown cells, 11% through the N_2-fixing system uncoupled to N_2 fixation, and 28% through the N_2-fixing system coupled to N_2 fixation.

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