Journal of Natural Products
Vol. 58, No. 9, pp. 1305-1314, September 1995

NOVEL TECHNIQUES FOR GROWTH CHARACTERIZATION AND PHYTOCHEMICAL ANALYSIS OF PLANT CELL SUSPENSION CULTURES[1]

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ABSTRACT.—Major problems associated with plant cell suspension cultures are the variability and instability of the cultures. Therefore, characterization of a suspension culture in every experiment is necessary to allow a comparison between different experiments. For this purpose, some new techniques were developed. A technique for growth characterization has been developed based on the dissimilation of the cultures. The main advantages of this method are that it is non-destructive, it supplies a dissimilation curve for each individual flask, and it is accurate. Here we present an accurate calculation for the curves and the most accurate way to measure them. Another technique was developed for the rapid quantification of the main organic intracellular components (especially carbohydrates and amino acids). Aqueous extracts are prepared and the components are quantified by ^{1}H-nmr spectroscopy. The main advantages of this method are non-selectivity and good sensitivity.

The developed techniques were applied in an experiment in which cultures growing on media containing different nitrogen levels were compared. The optimum quantity of nitrogen for growth and alkaloid production was found to be half the amount normally present in Murashige-Skoog medium.

Numerous studies have been carried out using plant cell suspension cultures. They are a potential source for the secondary metabolites produced by the parent plant. However, production of the desired compounds has usually turned out to be complex, as indicated in numerous reviews, such as that of Banthorpe (1). The main problems are that the desired compound is either not produced at all or is produced only in minor quantities, and that the cultures are usually unstable. Further, the production usually occurs only in a short period of culture growth and catabolism can influence the accumulation considerably.

Essential in the comparison of results obtained by various experiments, at various times, or by different groups, is the characterization of the suspension used and the conditions under which the experiment is performed. Important factors to be considered are: (a) the growth parameters of the culture, inclusive of inoculum density, maximum growth rate, biomass accumulation pattern, and uptake of nutrients; (b) the growth conditions, i.e., culture medium composition, gaseous exchange of the culture flasks, light, and temperature; and (c) accumulation of intracellular and extracellular metabolites. In the present paper we will present some methods which were developed to obtain the above-mentioned data routinely, without the necessity of a major investment of time.

DEVELOPMENT AND VALIDATION OF A NOVEL METHOD FOR GROWTH CHARACTERIZATION.—Plant cell suspension cultures are usually grown on media supplied with carbohydrates as carbon source. Based on the dissimilation of the cultures, a novel method was developed for growth characterization (2). The method is based on the idea that in the dissimilation process equimolar quantities of CO_2 are exchanged with O_2. This exchange will cause a loss of weight in the culture flask.

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[1]Invited paper presented at the Young Investigators Symposium at the International Congress on Natural Products Research, Halifax, Nova Scotia, Canada, 3 August 1994.

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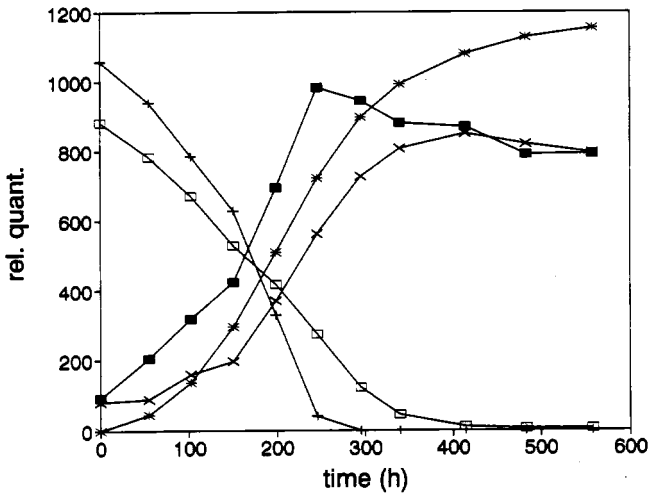

FIGURE 1. Comparison of different methods of growth characterization (time-course experiment): ■: dry wt (1000=1 g/flask); +: carbohydrates in the medium (1000=8 mmol monomer/flask); □: nitrate in the medium (1000=3 g/liter); *: loss of weight by dissimilation (1000=500 mg); X: fresh wt (1000=20 g/flask). The cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium.

In initial experiments, the validity of the model was demonstrated (2). The relationship with traditional methods of growth characterization has been determined repeatedly (e.g., Figure 1). It has been found that in sugar-limited cultures the dissimilation curves show an inflection at the moment the maximum dry weight is reached, which coincides with the point where the extracellular carbohydrates are depleted (2).

Each dissimilation curve is obtained by subtracting the evaporation from the loss of weight curve of a culture flask (Figure 2). The evaporation of each flask is estimated by using flasks without cells (blanks). The average evaporation of the blanks is subtracted

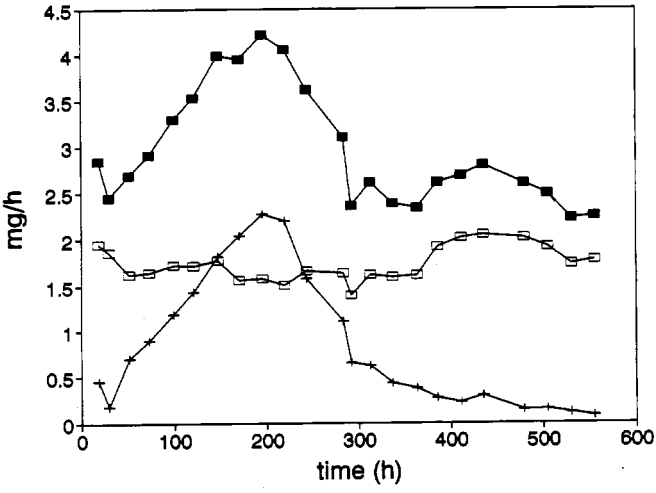

FIGURE 2. Calculation of the loss of weight by dissimilation (+). From the total loss of weight of a culture flask (■), the loss of weight by evaporation (□) is subtracted. In this example (from the time-course experiment), a subtraction factor of 1.22 was used. The method of obtaining the subtraction factor is discussed in the text. The dissimilation curves, as displayed in other figures, show the cumulative loss of weight by dissimilation.

from the total loss of weight of each culture flask after multiplication with a so-called subtraction factor. The subtraction factor reflects the relative porosity of the culture flask closure and usually shows little variation (values between 0.7 and 1.3), if all flasks are closed with the same type of closure.

In principle, three methods are available for the estimation of the exact value of the subtraction factor (2): (a) before the experiment: the evaporation relative to the blanks can be determined by weighing the flasks before the inoculation, but a prerequisite is that during inoculation the ratio between the evaporations should not be changed; (b) during the experiment: the factor is estimated based on the appearance of the dissimilation curve, but only when all the cells are dead will the exact subtraction factor be obtained; and (c) after the experiment: the subtraction factor can be obtained by calculating the loss of weight. Initially it was thought that setting up a carbon balance would be sufficient (2), but as will be explained below, for more accurate calculations an oxygen and hydrogen balance must also be set up.

The second method is the easiest and is sufficient for experiments in which the main interest is the comparison of the growth of cultures in individual flasks. To explore the possibilities of obtaining additional data, however, a more thorough investigation of the other methods has been made. Experiments were performed which focused on the calculation of the final loss of weight. In one such experiment, flasks were harvested at different points during the culture period. A dissimilation curve was obtained from each flask until the time of harvest. After harvesting, the sugar and nitrate content of the medium and the dry weight and elemental composition of the biomass were determined from each flask.

For each flask the amount of carbon in the different fractions was calculated. This resulted in the construction of a carbon balance for the experiment (Figure 3). The loss of weight by dissimilation calculated for each individual flask from this carbon balance

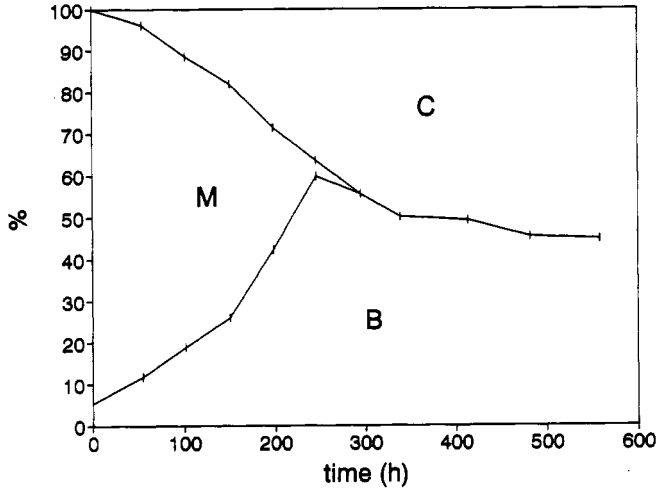

FIGURE 3. Carbon balance, displaying the distribution of carbon during a time-course experiment with a *Catharanthus roseus* suspension culture. Percentage of carbon in: sugars in the medium (M), the biomass (B), and CO_2 (C).

was about 20% below the value estimated from the appearance of the dissimilation curve. The most probable explanation for the observed difference was an imbalance between the O_2 used by the culture and the CO_2 produced. To test this hypothesis, an oxygen balance

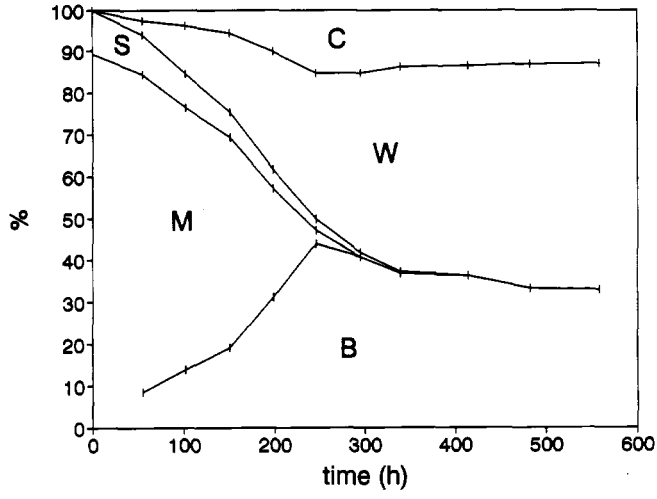

FIGURE 4. Oxygen balance, displaying the distribution of oxygen during a time-course experiment with a *Catharanthus roseus* suspension culture. Percentage of oxygen in: sugars in the medium (M), salts in the medium (S), the biomass (B), and H_2O (W). Fraction (C) is the fraction which leads to CO_2 as a result of an imbalance between the uptake of O_2 and the production of CO_2.

was also set up (Figure 4). In this balance the oxygen initially present in H_2O can be ignored. The initial amount of oxygen was present in extracellular sugars, the inoculum biomass, and in salts in the medium. The final amount of oxygen in salts in the medium was calculated by multiplying the initial amount with the percentage of the nitrate still present in the medium (nitrate contains >90% of the oxygen initially present in salts).

The amount of oxygen that was incorporated in H_2O was calculated from a hydrogen balance (Figure 5): all hydrogen which disappeared was considered to be incorporated into H_2O. The oxygen deficit in the oxygen balance was then considered to be the imbalance between the O_2 uptake and CO_2 production. When this amount was added to the already calculated carbon loss, the resulting dissimilation curves were very close

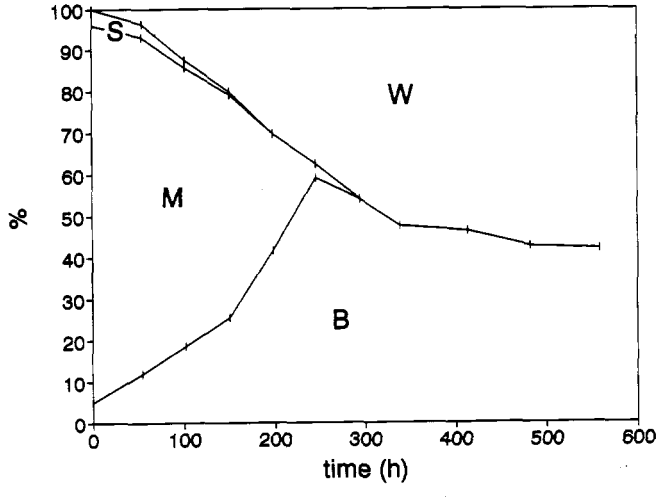

FIGURE 5. Hydrogen balance, displaying the distribution of hydrogen during a time-course experiment with a *Catharanthus roseus* suspension culture. Percentage of hydrogen in: sugars in the medium (M), salts in the medium (S), the biomass (B), and H_2O (W).

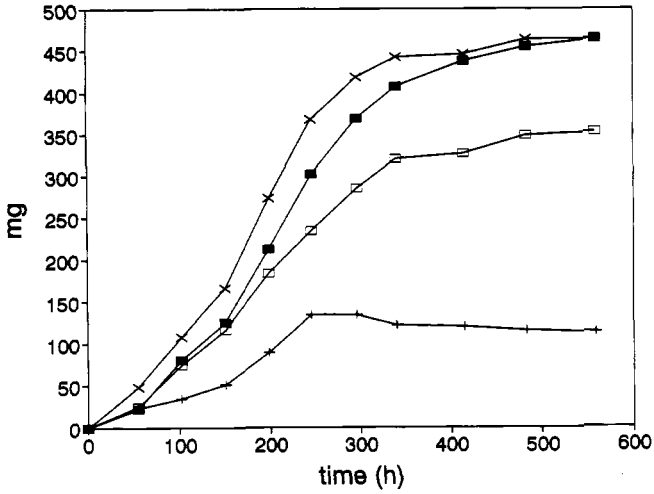

FIGURE 6. Calculated and experimental dissimilation curves. ■: experimental dissimilation curve; X: calculated dissimilation curve; □: loss of carbon (C in the carbon balance); +: oxygen deficit (C in the oxygen balance).

to the expected curves (Figure 6). Relatively large deviations were found only at the points where the dissimilation rate was highest, but as will be seen below, this could be due to the fact that a dissimilation curve is not equal to the actual CO_2 production curve, because the loss of weight only occurs after the CO_2 has left the flask.

MONITORING THE GAS EXCHANGE OF THE CULTURE FLASK.—From the dissimilation curves, the CO_2 levels inside the flask can be determined, and thus the difference between the dissimilation curve and the actual CO_2 production curve may also be determined. These data can be calculated if the rate constant for CO_2 diffusion has been calculated.

In a representative experiment, the dissimilation curves were determined using subtraction factors determined before the experiment began. The rate constants for CO_2 diffusion were also determined. To avoid the change of subtraction factors upon inoculation, flasks with two openings were used. One of the openings was closed with the normal silicone stopper, and the other was hermetically closed with a rubber stopper. The subtraction factors of individual flasks were determined by weighing all flasks during the two weeks before inoculation. After this, the inoculum was introduced through the opening which was closed by the rubber stopper (thus avoiding any change in the subtraction factors). A good correlation was found between the calculated and experimental values for the dissimilation.

The suspension culture was also harvested through the opening closed with the rubber stopper. The CO_2 diffusion rates were then determined. The flasks were filled with CO_2 gas and the rate of diffusion was determined. This experiment was performed with empty flasks and with flasks containing H_2O. With the determined values, the CO_2 levels inside the flask and the difference between the dissimilation curve and the actual CO_2 production curve were determined (Figure 7). Maximum CO_2 levels were calculated at between 8 and 12.5%. Those differences are considerable, taking into account that the variation between stoppers is quite small compared to other types of culture flask closures. Significant effects on growth were found in this experiment, but it is important to note that 500-ml Erlenmeyer flasks were utilized. In other experiments, carried out in 250-ml Erlenmeyer flasks, no effects on the growth rate were observed. Thus, it is possible, by the simple regular weighing of culture flasks, to obtain data on the growth of the culture and on the gaseous composition inside the culture flask.

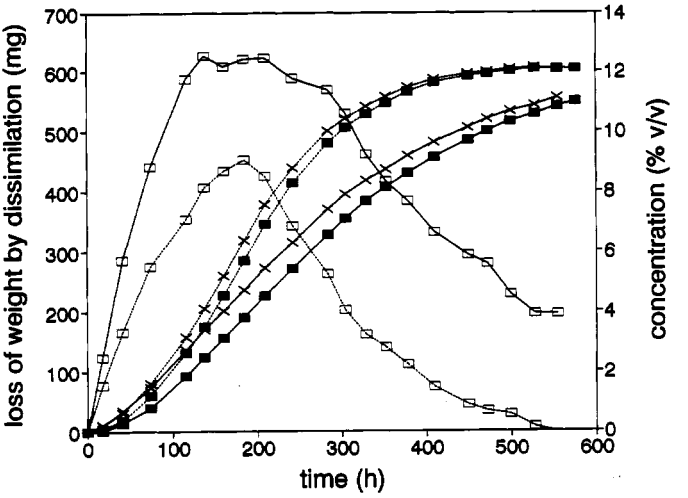

FIGURE 7. CO_2 levels inside culture flasks. The CO_2 levels (□), calculated CO_2 production curves (X), and experimental dissimilation curves (■) of the two flasks which differed most in their constants for CO_2 diffusion, 0.0136 h^{-1} (solid lines) and 0.0315 h^{-1} (dashed lines), respectively.

ACCUMULATION OF INTRACELLULAR AND EXTRACELLULAR METABOLITES.—Secondary metabolites are the compounds that are usually of main interest for the investigator dealing with plant cell cultures, e.g., indole alkaloids or terpenes. To quantify these compounds, specific chromatographic systems are usually needed.

The quantification of primary metabolites such as sugars and amino acids has been found to be very useful to monitor the physiological state of the culture. A method has been developed, which is based on the measurement of ^{1}H-nmr spectra of aqueous extracts of cell culture biomass, to quantify these major primary metabolites (3). Sugars were found to accumulate during the growth of the culture as long as extracellular carbohydrates were present. The maximum level of intracellular carbohydrates was found at the moment the extracellular carbohydrates were depleted (4).

Further, several amino acids are usually present in quantities high enough to be detected, e.g., alanine, arginine, asparagine, glutamine, phenylalanine, threonine, and valine. Two types of time-course for the intracellular components were found: (a) compounds with the highest levels during growth; and (b) compounds with the lowest levels during growth. In *Catharanthus roseus* cell cultures the sugars and glutamine belong to group 1, while the other amino acids belong to group 2. Also the indole alkaloids belong to group 2, thus only in the stationary phase is accumulation of these compounds observed.

Despite the fact that two types of time-course can be distinguished, each compound shows a specific time-course. Variations occur at the moment accumulation or depletion starts and the levels which are maintained. The complete spectrum thus gives a good characterization of the cell material and the stage of growth.

THE INFLUENCE OF DIFFERENT NITROGEN LEVELS IN THE MURASHIGE-SKOOG MEDIUM ON GROWTH AND METABOLITE PRODUCTION.—In an experiment, cells from *C. roseus* were grown on Murashige-Skoog media containing different levels of nitrogen (respectively 100, 75, 50, and 25% of the normal amount). The time-courses of dry weight and dissimilation showed faster growth in the medium with decreasing nitrogen concentrations (Figure 8). Also, the ajmalicine accumulation started earlier in those media with lower nitrogen, but in the medium with 25% nitrogen, hardly any accumulation

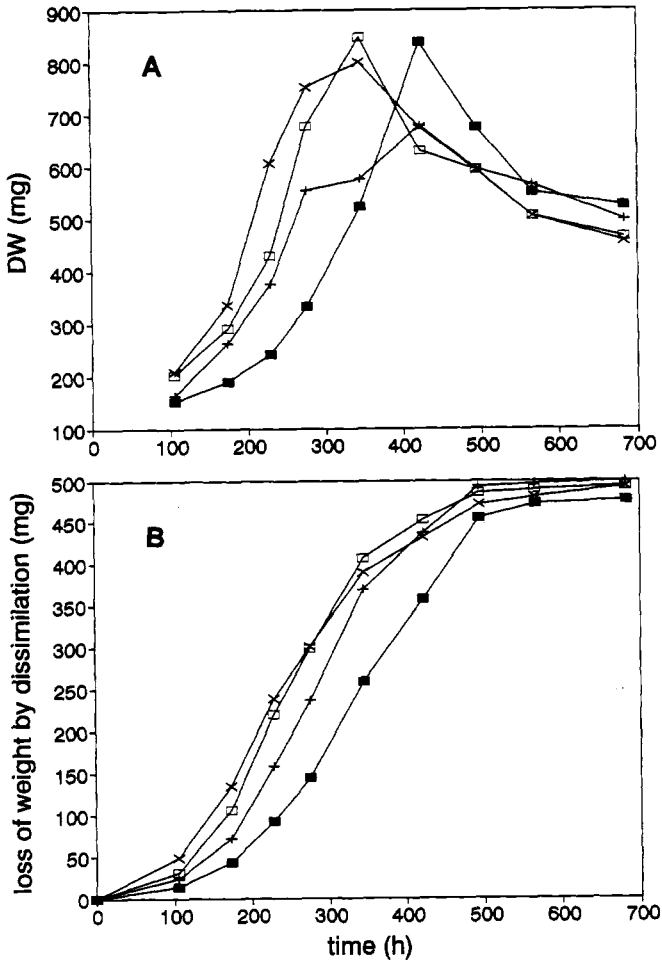

FIGURE 8. Time-courses of dry wt (A) and dissimilation (B) of the cultures grown on media containing different amounts of nitrogen, 100% (■), 75% (+), 50% (□), and 25% (X), respectively, of the amount of normal MS medium.

occurred (Figure 9). In the medium with 50% nitrogen, the accumulation started about 150 h earlier than on the normal medium, and high levels were attained. The actual moment the ajmalicine accumulation begins might be influenced by intracellular glutamine levels (Figure 10). Also, the rapid disappearance of ajmalicine, which may be related to the exhaustion of the metabolite pools, should be noted.

In cultures in medium with lower nitrogen the increased speed of dry weight accumulation corresponds with the faster accumulation of intracellular carbohydrates (Figure 11). Initially, mainly glucose was accumulated, while later large amounts of sucrose were accumulated.

The accumulation of a number of other components could also be monitored, e.g., alanine, arginine, choline, phenylalanine, and valine. All of these showed, in principle, type (b) behavior.

EXPERIMENTAL

CELL CULTURES.—Cell suspension cultures of *Catharanthus roseus* were used. In the various experiments, different cell lines were used, as indicated in the experimental description. All lines produced indole alkaloids and were green. Line 9CR58 was initiated in 1988 on hormone-free MS medium (5), supplemented

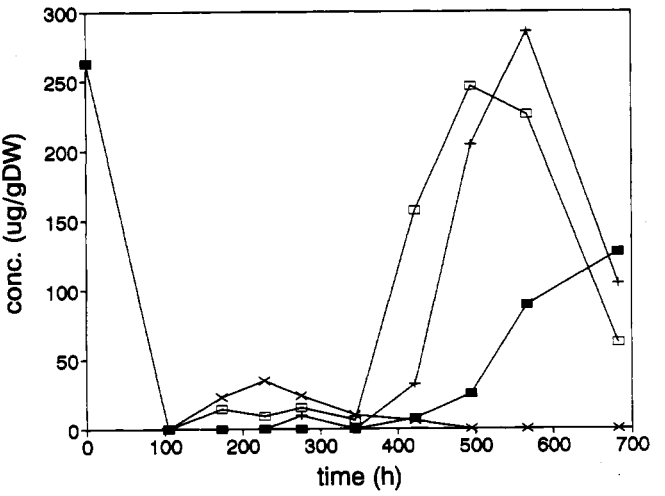

FIGURE 9. Ajmalicine accumulation of the cultures grown on media containing different amounts of nitrogen, 100% (■), 75% (+), 50% (□), and 25% (X), respectively, of the amount of normal MS medium.

with 30 g/liter sucrose, from another line that was maintained on LS medium (6), supplemented with 2 mg/liter NAA, 0.2 mg/liter kinetin, and 30 g/liter sucrose, and which was initiated in 1981. Line A12A1 was derived from 9CR58 by separate subculturing. Line 11CR9/58 was derived from A12A1 in 1990 by subculturing on a Gamborg B5 medium (7) supplemented with 1.86 mg/liter NAA and 20 g/liter sucrose. All flasks were closed with Silicosen T-32 stoppers (Shin-Etsu Polymer Co. Ltd., Japan).

TIME-COURSE EXPERIMENT.—Twenty 250-ml Erlenmeyer flasks, each containing 50 ml of medium, were inoculated with an accurately weighed amount of cells (±3 g), obtained by rapid aseptic filtration of a culture (line A12A1) over a glass filter. For the determination of dissimilation curves, seven blanks were used to determine evaporation. Every two to three days two flasks were harvested. The pH, fresh wt, dry wt, and elemental composition of the biomass, and the sugar and nitrate content of the medium were determined.

EXPERIMENT TO MONITOR GASEOUS EXCHANGE.—Eight 500-ml flasks with two openings, each containing 100 ml of medium, were inoculated with an accurately weighed amount of cells (±7 g), obtained by rapid aseptic filtration of a culture (line 11CR9/58) over a glass filter. For the determination of

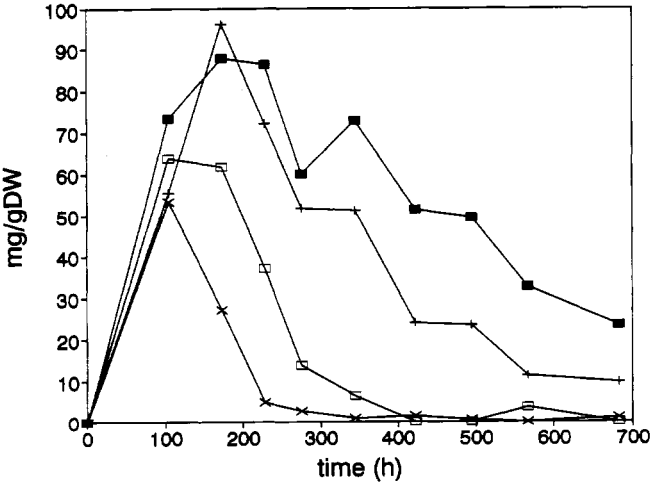

FIGURE 10. Intracellular glutamine of the cultures grown on media containing different amounts of nitrogen, 100% (■), 75% (+), 50% (□), and 25% (X), respectively, of the amount of normal MS medium.

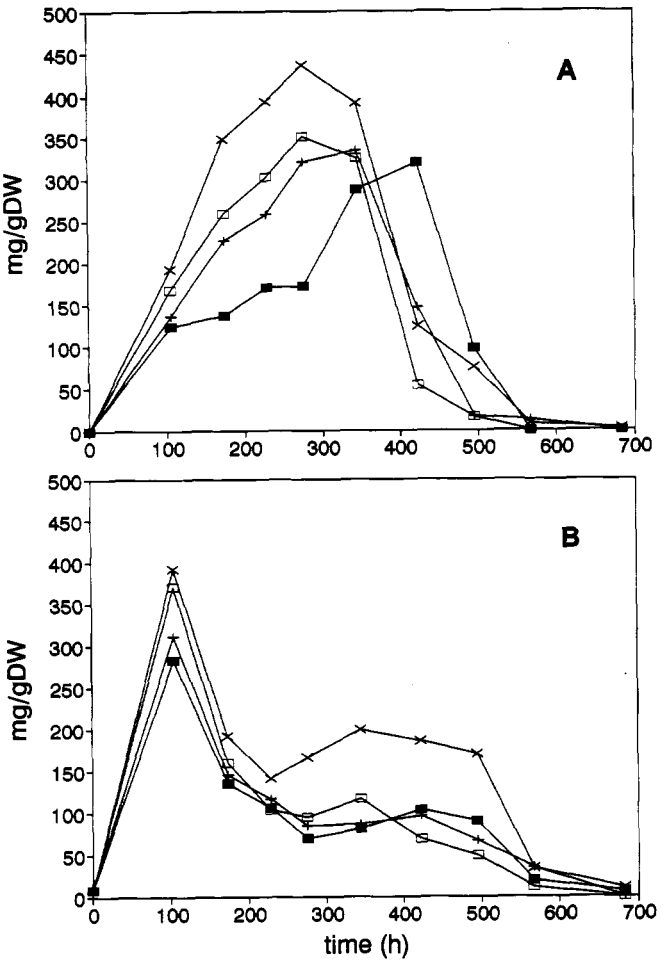

FIGURE 11. Intracellular carbohydrates of the cultures grown on media containing different amounts of nitrogen, 100% (■), 75% (+), 50% (□), and 25% (X), respectively, of the amount of normal MS medium. In A, the intracellular sucrose levels are shown and in B, the intracellular glucose levels.

dissimilation curves, six blanks were used to determine the evaporation. For determination of the rate constants of CO_2 diffusion, the flasks were filled with CO_2 gas, and placed on the shaker. The flasks were weighed regularly. The loss of weight curve was fitted on the equation: $Y = max - max*10^{-kt}$ in which k is the rate constant.

EXPERIMENT TO INVESTIGATE DIFFERENT NITROGEN LEVELS.—Eighty 250-ml Erlenmeyer flasks (four series of 20 flasks), each containing 50 ml of medium, were inoculated with an accurately weighed amount of cells (±4 g), obtained by rapid aseptic filtration of a culture (line 9CR58) over a glass filter. For the determination of dissimilation curves, ten blanks were used to determine the evaporation. Every two to five days two flasks of each series were harvested.

DISSIMILATION CURVES.—Dissimilation curves were obtained by regular weighing of the culture flasks on a Sartorius L420P+ balance (range 420 g; accuracy 1 mg). The weighing data were processed using a specialized computer program, which may be obtained upon request from the authors.

NMR ANALYSES.—The nmr analyses were performed as described previously (3).

ACKNOWLEDGMENTS

We wish to thank Dr. H.J.G. ten Hoopen and Dr. G. van der Steen for the elemental analyses.

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Received 17 January 1995