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Effect of nitrogen supplementation on the longevity of antibiotic production by immobilized cells of *Penicillium urticae*

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Summary. Conidia of *Penicillium urticae* were immobilized in Kappa-Carrageenan beads (2–3 mm) by a previously described procedure to yield an in situ grown immobilized cell population which could be induced to produce the antibiotic and mycotoxin, patulin. When repeatedly transferred into a nitrogen-free production medium every 2 days, the patulin productivity of these cells gradually decreased to 50% within 14 days while the total cell protein remained constant. This decline was due to the gradual loss of the cells' catalytic capacity for converting glucose to 6-methylsalicylic acid (6-MSA), the first metabolite of the patulin pathway, as well as for converting 6-MSA to patulin. When these 14 day-old cells were incubated in a nutrient rich growth medium for 2 days their patulin producing activity increased from 50% to 130%. On the other hand the addition of a protein synthesis inhibitor, cycloheximide, to the N-free production medium drastically reduced the patulin producing activity of the immobilized cells; in particular, their capacity for converting 6-MSA to patulin. The cells' patulin producing activity was maintained at > 100% for longer than 15 days when the cells were repeatedly transferred into a yeast extract supplemented production medium or when they were occasionally transferred into 10 or 20% strength growth medium. Repeated transfers to a 10% strength growth medium appeared to stabilize the cells' capacity for converting 6-MSA to patulin.

Introduction

Antibiotics are generally referred to as "secondary metabolites" because these compounds are not essential for the exponential growth of producing cells. In most microorganisms the pathways of antibiotic biosynthesis involve several steps and these pathways are clearly distinct from the ones responsible for the synthesis of common cell constituents (Malik 1982). Thus, the onset of an antibiotic biosynthesis is preceded by the transcription of several genes and the subsequent translation of m-RNAs (Gallo and Katz 1972). A continued synthesis of specific enzyme proteins also appears to be essential for continued antibiotic synthesis (Marshall et al. 1968; Gaucher et al. 1981). Although the pathways are distinct, antibiotics are generally produced from the same intermediary metabolites which are required for active cell growth (Martin and Liras 1981). Thus, biosynthesis of an antibiotic involves two steps, first the conversion of nutrients (glucose, yeast extract, etc.) to intermediary metabolites and then conversion of these primary metabolites to the antibiotic molecule. Hence many catabolic functions are as important for antibiotic biosynthesis as they are for cell growth (Vanek and Mikulik 1978).

Once derepressed and/or induced, the rate of antibiotic biosynthesis rapidly increases to a maximum value and then gradually decreases to the preinduced level (Queener and Swartz 1979). The precise mechanism of this cessation is not known, although three major reasons can be cited (Martin and Demain 1980). One is a depletion of intermediary metabolite precursors due to a diminishing availability of nutrients and/or a decline in the cells' catabolic activities (Drew and Demain 1977). Another is feedback inhibition of secondary metabolism enzymes by end-product(s) (e.g., antibiotics and their pathway intermediates). The third reason is the loss of one or more pathway enzymes due to the inherent instability of these enzymes, their degradation by intracellular proteases and/or lack of de novo enzyme synthesis. To date the latter reason appears to be the principal factor (Gaucher et al. 1981). Thus, the longevity of antibiotic production may be improved

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Table 1. Composition of liquid media for *P. urticae* (g/L)

Ingredients	Growth medium	Production media			
	Complete medium[a]	20% Complete medium	10% Complete medium	Yeast-extract (Y.E.) medium	Nitrogen-free (N-free) medium
KH_2PO_4	13.6	13.6	13.6	13.6	13.6
Glucose	50.0	25.0	25.0	25.0	25.0
Yeast extract	5.0	1.0	0.5	1.25	–
Citric acid (anhydrous)	9.8	1.96	0.98	–	–
Na_2SO_4 (anhydrous)	1.0	0.2	0.1	–	–
Trace metal solution[b]	10.0 ml/L	2.0 ml/L	1.0 ml/L	–	–
pH	6.5	6.0	6.0	6.0	5.6

[a] GrootWassink and Gaucher (1980)
[b] Yamamoto and Segel (1967)

by increasing the inherent and/or proteolytic stability of antibiotic specific enzymes and by promoting continued de novo enzyme synthesis. A continuous but controlled addition and removal of nutrients and products may also be indirectly of value.

The stability of intracellular enzymes appears to be improved by the immobilization of cells (Klibanov 1983). We have reported that, in comparison to free cells, immobilized cells of *Penicillium urticae* exhibited a three-fold greater half-life for patulin production, when such cells were repeatedly transferred into a nitrogen deficient production medium (Deo and Gaucher 1983). It was, however, not clear whether this increase in the half-life was due to the retention of the catabolic activities of cells or due to the retention of patulin pathway enzymes. There is some indication that the latter is most probable (K. S. Lam, unpublished results). In either case a replenishment of amino acids (i.e., nitrogenous nutrients) to offset their loss during protein turnover might be of value. Hence, we determined the effect of supplying these cells with a limited amount of a nitrogenous nutrient. Both the half-life of patulin production and the "patulin pathway's efficiency" (i.e., capacity for converting 6-MSA to patulin) were examined. The contrasting effect of inhibiting de novo protein synthesis, on these two parameters, was also examined.

Methods

Cultivation procedures. Penicillium urticae strain NRRL 2159A was maintained and cultivated by using previously described media and procedures (GrootWassink and Gaucher, 1980).

The cells were routinely immobilized in k-Carrageenan (Sigma) beads by the in situ grown immobilization procedure described by Deo and Gaucher (1983). The ~3 mm beads (350–400) containing conidia were shaken (280 RPM, 2.5 cm stroke) at 28° C in 50 ml of complete growth medium (Table 1) in a 500-ml Erlenmeyer flask for 36 h. These beads were then used for the semi-continuous production of patulin in various production media.

The semi-continuous production of patulin by immobilized cells was achieved by washing 36 h old, filtered, in situ grown immobilized cells with three 150 ml volumes of sterile phosphate buffer (0.1 M, pH = 7.0) and then resuspending into 50 ml of N-free or yeast-extract medium (Table 1). This was repeated every 48 h for the N-free medium and every 24 h for the yeast extract medium. Culture broth samples (5–8 ml) were collected before every transfer. In one experiment 100 μg/ml of cycloheximide was added to the N-free medium at the beginning of the first and all subsequent 48-h transfers, the second and all subsequent 48-h transfers or the third and all subsequent transfers. This concentration of cycloheximide was 20-fold greater than that required to totally inhibit net protein synthesis in a free cell culture (GrootWassink and Gaucher, 1980). An excess of this protein synthesis inhibitor was added to ensure that its concentration at the periphery as well as at the centre of each bead was sufficient to inhibit all intracellular protein synthesis.

Diluted (20% or 10%) complete media (Table 1) were used to revive the patulin producing activity of cultures which had been repeatedly transferred into N-free medium. This was achieved by occasionally transferring the cells into these relatively nutrient rich media as will be described later.

Sampling procedures. After measuring the pH, the culture broth samples collected at the end of each transfer were filtered through a millipore (0.45 μm) filter and then stored at −20° C. These samples were used later for glucose and patulin assays. For high pressure liquid chromatographic (HPLC) analyses, these samples were processed as follows. Two milliliters of a filtered sample was extracted twice with 2.5 ml of ethyl acetate after adjusting the pH to 2.0 with two drops of 4 N HCl. The combined ethyl acetate extract was dried over about 0.5 g of anhydrous sodium sulfate and then concentrated at 30° C in a 15-ml conical glass centrifuge tube using a vortex vacuum evaporator (vortex speed 4.5, vacuum 2.5 in. Hg, Buchler model 3-2200). The concentrated samples were stored at −20° C.

For the estimation of total cell protein in a shake culture, beads from the entire culture were collected by filtration and washed with 150–200 ml distilled water. These beads were then freeze-dried for 24 h and stored at −20° C.

Assay procedures. The concentrations of glucose and patulin in the millipore filtered culture broth samples were determined by using previously described glucostat and bioassay procedures respectively (GrootWassink and Gaucher, 1980).

The quantitative estimation of various patulin pathway metabolites in the ethyl acetate extracts of culture broth samples was carried out by using a Hewlett-Packard high pressure liquid chromatograph (Model 1084B). Concentrated ethyl acetate

extracts were dissolved in 1.0 ml of HPLC grade methanol (Fisher). An aliquot (10 µl) of this sample, representing 20 µl of the original culture filtrate, was injected into a 4.6 × 200 mm, Merck RP-8, reverse phase column (particle size 10 µm). The gradient elution program for the effective separation of patulin pathway metabolites was the same as described by Jones et al. (1983).

The total cell protein in 100–200 mg of powdered freeze-dried beads was determined by using the Folin-Lowry protein assay procedure as previously described (Deo and Gaucher, 1983).

Results

Effect of a N-free production medium.

Suzuki and Karube (1979) have reported that when immobilized *Bacillus* sp. cells were repeatedly incubated in a production medium containing peptone and meat extract, their bacitracin producing activity was much higher than that of cells repeatedly incubated in a production medium containing only starch. After two successive 4-h incubations in a starch medium the bacitracin production decreased by 60%, while no decrease in the production occurred after two 4-h incubations in the peptone-meat extract medium. We have also observed a similar decrease (100% to 50%) in the patulin productivity of immobilized *Penicillium urticae* cells over a 14-day period, when these cells were transferred every 2 days into a N-free medium (Fig. 1). When these immobilized cells were given a 2-day "vacation" in complete medium after having spent 14 days in N-free medium, their patulin producing activity increased from 50% to 130% of the initial patulin production. Despite this regeneration, patulin production remained high for only one transfer before again declining to 60% over the next three transfers into N-free medium. The yield of patulin from glucose, however, remained constant despite changes in total patulin production and in medium composition. This indicates that glucose utilization declined along with patulin production and that the two processes are closely linked. The reactivation of patulin production by a 48-h incubation in complete medium may be due to a renewed synthesis of enzymes required for the catabolic utilization of glucose and/or for the biosynthesis of patulin from its precursors (Gaucher et al. 1981). Hence, the continued or intermittent de novo synthesis of key enzymes may be the key to continued patulin production.

Effect of cycloheximide.

Figure 2 shows the effect of a protein synthesis inhibitor, cycloheximide, on the activity of two consecutive sections of the glucose to patulin transformation by immobilized cells in N-free medium. The first section involves the catabolism of glucose to acetyl- and malonyl-CoA which are then used by a large synthetase to produce the first committed metabolite of the patulin pathway, 6-methylsalicylic acid (6-MSA). The second section involves the transformation of 6-MSA to patulin in a minimum of 9 steps (Gaucher et al. 1981). Given the stoichiometry of this latter section and the lack of accumulation of any pathway intermediates other than 6-MSA, the productivity of the first section is indicated by the total 6-MSA produced; that is by the "total patulin plus 6-MSA" produced from glucose (Fig. 2). Assuming that the re-entry of 6-MSA into the cells is not rate limiting (Gaucher et al. 1983), the productivity of the

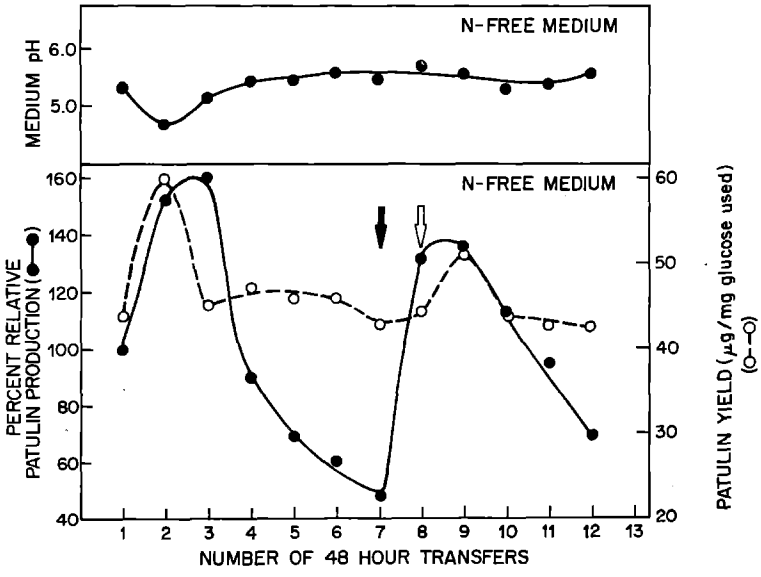

Fig. 1. Regeneration of the patulin producing activity of immobilized *P. urticae* cells. Immobilized cells were transferred into 50 ml of complete medium (↓) (Table 1) after the relative patulin production in N-free medium (Table 1) decreased to about 50% of its initial value of 0.71 mg/ml. After spending 48 h in this complete medium (⇓) these cells were washed with phosphate buffer (0.1 M, pH = 7.0) and once again repeatedly transferred into N-free medium every 48 h

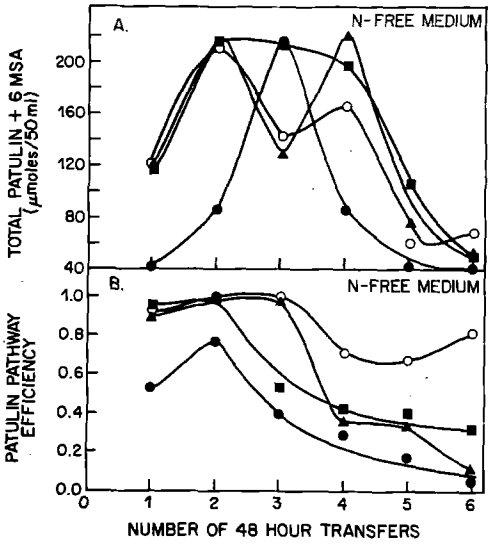

Fig. 2A, B. Effect of cycloheximide on the production of 6-MSA from glucose and on the efficiency of the patulin pathway during each 48-h period. Four separate 50-ml shake cultures of immobilized *P. urticae* were repeatedly transferred into N-free medium as usual. Cycloheximide (100 μg/ml) was added to one culture at the beginning of the first and all subsequent 48-h periods (●), to another at the beginning of the second and subsequent periods (■), and to a third at the beginning of the third and subsequent periods (▲). No cycloheximide was added to the fourth culture (○). Pathway efficiency is "total patulin produced per total 6-MSA (i.e., Patulin + 6-MSA) produced" by cells

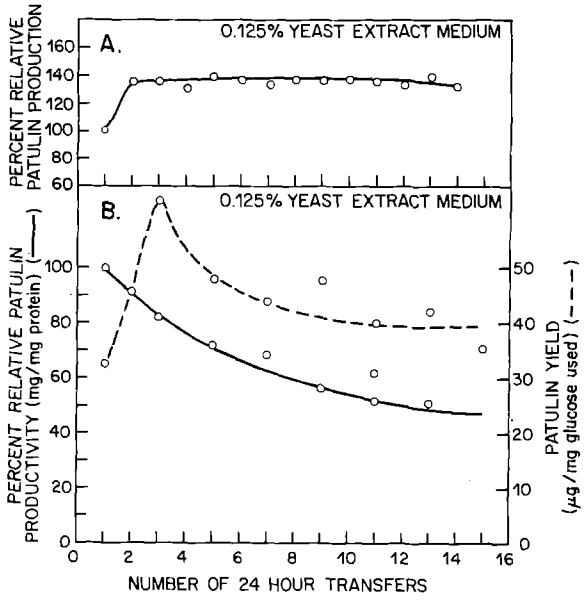

Fig. 3A, B. Effect of yeast extract on the longevity of patulin production by shake cultures of immobilized *P. urticae* cells. Immobilized cells were transferred every 24 h into replacement medium containing 0.125% yeast extract (Table 1). Patulin production (100%) equaled 0.437 mg patulin/ml, while patulin productivity (100%) equaled 0.31 mg patulin/mg cell protein

second section (i.e., the "patulin pathway efficiency") is indicated by the ratio "total patulin produced per total 6-MSA produced". Note that pathway metabolites accumulate in the culture medium with only negligible amounts present inside the cells (Gaucher et al. 1983), these metabolites are not degraded to any measurable extent (Neway and Gaucher 1981), and that all significant pathway intermediates and side products can be quantitatively assayed for by HPLC (Lam 1981).

Figure 2A suggests that in the short term (i.e., 2d) new enzyme synthesis is important for the conversion of glucose to 6-MSA (i.e., the first section) in N-free medium, but that thereafter the cycloheximide mediated inhibition of protease production may lead to the improvement of this conversion. This suggests that during long term incubation in N-free medium the levels of 6-MSA synthetase and/or relevant primary metabolism enzymes are more strongly affected by protease action than by new enzyme synthesis. Figure 2B shows that unlike the uninhibited control in which pathway efficiency remained high throughout 12 days in N-free medium, cycloheximide inhibited cultures increased or maintained their pathway efficiency for only about 2–4 days before suffering a drastic loss in this efficiency. These results suggest that in the short term any lack of new enzyme synthesis is offset by less protease synthesis and hence by a reduction in the loss of existing enzymes. In the longterm, however, the conversion of 6-MSA to patulin requires new enzyme synthesis. This inhibition study suggests that continued enzyme synthesis is more important for the biosynthesis of patulin from 6-MSA than for the biosynthesis of 6-MSA from glucose.

Effect of yeast extract supplementation

A continued, though limited, net intracellular protein synthesis could be accomplished either by the repeated transfer of cells into a 0.125% yeast extract medium or by occasional transfer into diluted (20% or 10%) complete media (Table 1). Figure 3A shows that the total production of patulin, at the end of each 24-h transfer into a dilute yeast extract medium, remained constant over 15 days while the yield and productivity decreased gradually (Fig. 3B). This implies that a continuous increase in the total mycelial protein occurred in these cultures with only a small fraction of this protein contributing directly or indirectly to patulin biosynthesis, and that during each successive incubation in yeast extract medium, a larger portion of the consumed glucose was directed

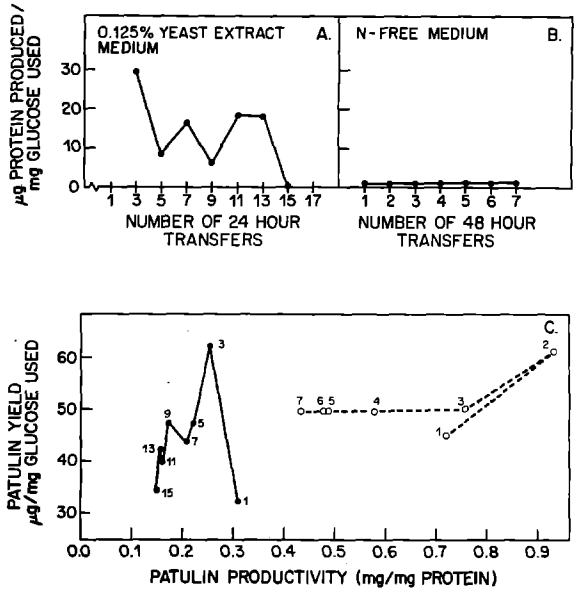

Fig. 4. The yield of cell protein from glucose (**A, B**) and the relationship between the patulin yield and productivity (**C**) during repeated transfers of immobilized *P. urticae* cells into replacement media. Cells were transferred into either 0.125% yeast extract medium (●) or into N-free medium (○) every 24 h (A) or 48 h (**B**) respectively. The arabic numbers adjacent to each point (**C**) represent the number of transfers into the respective medium

away from patulin biosynthesis and towards biomass production.

A comparison of the behavior of immobilized cells transferred into 0.125% yeast extract versus N-free medium is presented in Figure 4. Plots of yield versus productivity for transfers into 0.125% yeast extract versus N-free medium (Fig. 4C) shows that patulin production generally decreases with an increasing number of transfers in both cases, but that the range of productivity is higher for N-free medium. The yield, after an early increase drops in the yeast extract medium but remains constant in the N-free medium. Since cells transferred to the yeast extract medium produce as much patulin (i.e., 0.6 mg/ml) in 24 h as cells transferred to N-free medium produce in 48 h (i.e., 0.63 mg/ml), it is clear that changes in protein synthesis (i.e., biomass) and in glucose utilization are the important parameters. Thus in the yeast extract medium, the cultures' total protein content increased dramatically from 70 to 200 mg per 50 ml of culture in about 12 days and this was accompanied by the use of 8.22 g of glucose. In contrast, the culture in the N-free medium showed virtually no change in its total protein content (i.e., −2 mg) and over 12 days utilized about half the amount of glucose (4.83 g). This is illustrated in Fig. 4A and B in which the amount of protein produced/mg glucose used is plotted. Clearly, the N-free medium was superior because growth was limited. This provided a higher productivity and a constant yield (Fig. 4C) and maintained the integrity of the beads. In yeast extract medium not only were the productivity and yield lower, but the physical stability of the beads was significantly less. The cells in yeast extract medium did, however, produce about twice as much patulin in the same period of time (i.e., a net of 8 versus 5 mg/ml over 14 days). This suggested that a compromise nutrient protocol which limited increase in biomass while promoting the activity of the glucose to patulin pathways should be sought.

Effect of diluted complete growth medium

Figure 5 shows that after four transfers into N-free medium patulin production dropped as before (Fig. 1). A single transfer into 20% complete medium arrested this decline and reestablished a high patulin production. Three subsequent transfers into N-free medium (i.e., the 6th−8th transfers) again led to a decline. The use of 10% and 20% complete media for the 9th and 11th transfers respectively gave a similar recovery and subsequent decline. The degree of the recovery was proportional to the strength (20% or 10%) of complete medium used. The yield of patulin from glucose showed a high degree of correlation with the relative patulin production, especially when the cells were activated by being transferred into 10% complete medium. Finally, towards the end of this 36-day experiment, repeated transfers into 10% complete medium provided a steady production and yield of patulin. This was similar to the patulin production when cells were repeatedly transferred to 0.125% yeast extract medium (Fig. 3). But in contrast to this latter experiment, cells incubated in 10% complete medium also showed a steady rather than a declining yield of patulin from glucose. Note that this 10% complete medium contained 0.05% yeast extract while the 0.125% yeast extract medium did not contain any of the other ingredients of the complete medium except for phosphate buffer and glucose (Table 1).

A further analysis of the experiment described in Fig. 5 shows that "total 6-MSA" productivity (Fig. 6A) as well as yield of "6-MSA from glucose" (Fig. 6B) increased during the initial three transfers into N-free medium but declined during the fourth transfer. Occasional transfers into 20% (5th and 11th) of 10% (9th) and repeated transfers into 10% complete medium (14th and 18th) generally stimulated total 6-MSA production and improved its yield from glucose. The degree of this stimulation was

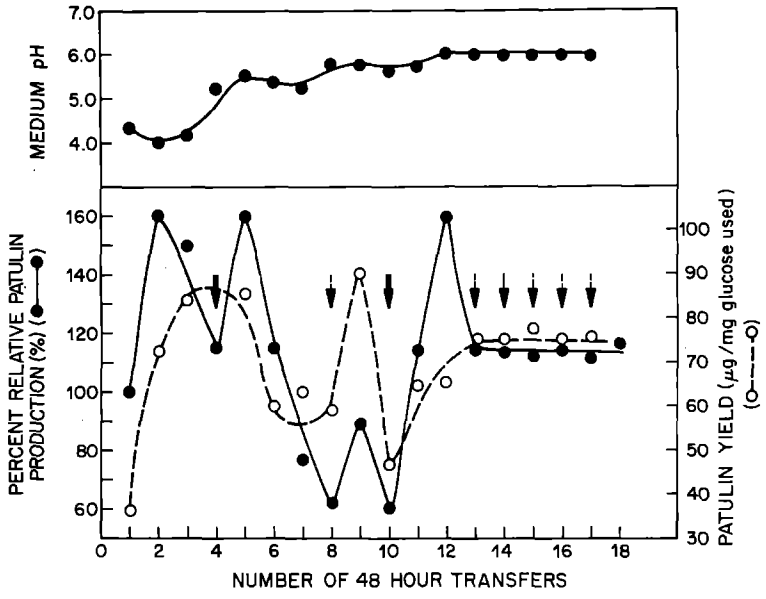

Fig. 5. Effect of complete media of different strengths on the longevity of patulin production by shake cultures of immobilized *P. urticae*. Immobilized cells were transferred into 50 ml of fresh N-free medium every 48 h except as stated below. The 5th and 11th transfers (↓) were made into 20% complete medium while the 9th, 14th, 15th, 16th, and 17th transfers (⇣) were made into 10% complete medium (Table 1)

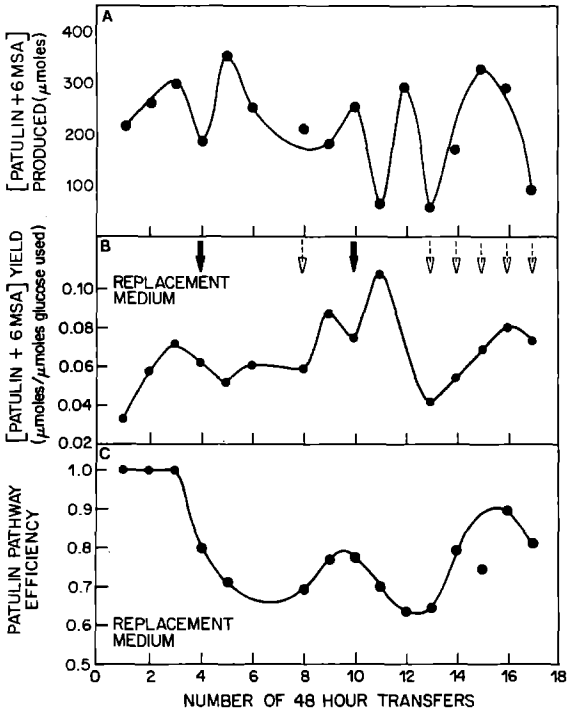

Fig. 6. Effect of occasional transfers into diluted media on the total production of patulin + 6-MSA (**A**), the yield of patulin + 6-MSA (**B**) and the patulin pathway efficiency (**C**) in shake cultures. Immobilized cells were transferred into N-free medium, 20% complete medium (↓) or 10% complete medium (⇣) as described in Fig. 5. Pathway efficiency is defined in the legend to Fig. 2.

generally proportional to the strength of the medium (20% to 10%).

In the case of the 5th, 9th, and 11th transfers, the expected increases were inexplicably delayed by 48 h or one transfer. Figure 6C shows three different patterns of "pathway efficiency" for the conversion of 6-MSA to patulin in this experiment. A very high (90%) efficiency during the first three transfers into N-free medium was followed by a significant decline to less than 70% even when 20% complete medium was used in the 5th and 11th transfers. A more dilute 10% strength medium led, however, to a recovery in efficiency (i.e., 80%) which was sustained during the last five transfers to this medium. Again the more growth promoting medium appears to at least negate the expected positive effect on the 6-MSA to patulin conversion.

The high pathway efficiency of cells during the first three transfers into N-free medium was also accompanied by an increase in the relative patulin production from 100% to 160% (Fig. 5). However, during the course of each of these three 48 h incubations in N-free medium, the pathway efficiency was not uniformly 90% (Fig. 7, panels 1, 2, and 3). Instead the efficiency increased markedly to this value during an approximately 12-h period which began at 12–24 h into the 48-h incubation. Furthermore, as the cells were repeatedly exposed to this N-free medium during the first three transfers, the pathway efficiency at 12 h of incubation increased from 40% to 75% (Fig. 7, panels 1, 2, and 3). This increasingly rapid recovery upon transfer into fresh N-free medium was arrested during the fourth transfer which showed a decline in efficiency. An increase in efficiency which after 48 h exceeds that achieved at the end of the previous incubation did not occur in N-free (Fig. 7, panels 10, 12, and 13) or in 20% complete medium (Fig. 7, panels 5 and 11). An increase was observed, however, when the cells were transferred into 10% complete medium (Fig. 7, panels 9, 14–17).

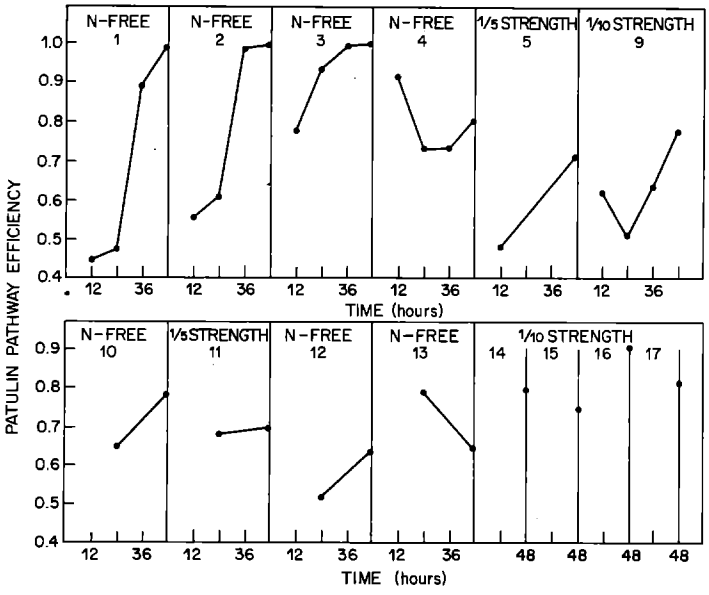

Fig. 7. The patulin pathway efficiency of *P. urticae* cells during the course of each transfer into N-free or diluted complete medium. Immobilized cells were transferred every 48 h into N-free medium, 20% complete medium ($^1/_5$ strength) or 10% complete medium ($^1/_{10}$ strength) as described in Fig. 5. Pathway efficiency is defined in the legend of Fig. 2

Discussion

In *Penicillium urticae* the onset of patulin production is preceded by the synthesis of specific mRNAs (Deo and Gaucher, in preparation) which are responsible for the synthesis of patulin pathway enzymes (Gaucher et al. 1981). Thereafter, the net pool of active enzymes is determined not only by a continued synthesis but also by enzyme degradation. A reduced synthesis and an increased rate of degradation, probably leads to the cessation of patulin production. Thus the regeneration of the patulin producing activity after a 48-h "vacation" in complete medium (Fig. 1) suggests that after seven transfers into N-free medium an external source of an assimilable nitrogen is essential to promote the synthesis of "patulin synthetases" and thus to prevent the loss of patulin producing activity due to degradation or inactivation.

The contrasting effects of the protein synthesis inhibitor, cycloheximide, on the two consecutive sections of patulin biosynthesis (Fig. 2) indicate that the conversion of glucose to 6-MSA is most sensitive to the action of proteases while the conversion of 6-MSA to patulin has a greater requirement for new enzyme synthesis. A transient level of m-RNAs and a large proteolytic target such as 6-MSA synthetase would be in keeping with this conclusion (Lynen et al. 1978). Thus an efficient and preferential conversion of glucose to patulin requires a nutritional state which protects and replenishes the necessary enzymes and which does not stimulate a rapid accumulation of biomass from glucose nor represses the expression of secondary metabolism genes.

The high patulin producing activity, constant yield of patulin from glucose, and high patulin pathway efficiency during the first three transfers into N-free medium (Figs. 5 and 6) indicate that the intracellular levels of the various enzymes responsible for patulin biosynthesis were temporarily well balanced in this nutritional environment. The loss of this balance during the fourth transfer may be attributed to declining m-RNA and enzyme synthesis and/or increasing proteolysis. This balance could not be re-established by the supplementation of only inorganic nutrients (Deo and Gaucher, 1983), organic nitrogen sources (Fig. 3) or by occasional "vacations" into a growth sustaining medium (Figs. 1 and 5). It could be regained, however, when a continued and controlled supply of both organic and inorganic nutrients was maintained (Figs. 5 and 6). This study shows that a 10% strength complete medium provides such an optimum nutrient environment.

In summary, this study shows that the longevity of antibiotic production by immobilized cells can be improved by manipulating the composition of the production medium and the recycle protocol. These manipulations should be generally directed towards maintaining balanced intracellular levels of antibiotic biosynthetic enzymes.

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References

Deo YM, Gaucher GM (1983) Semi-continuous production of the antibiotic patulin by immobilized cells of *Penicillium urticae*, Biotechnol Lett 5: 125–130

Drew SW, Demain AL (1977) Effect of primary metabolites on secondary metabolism. Ann Rev Microbiol 31: 343–356

Gallo M, Katz E (1972) Regulation of secondary metabolite biosynthesis: Catabolite repression of phenoxazinone synthetase and actinomycin formation by glucose. J Bacteriol 109: 659–667

Gaucher GM, Lam KS, GrootWassink JWD, Neway J, Deo YM (1981) The initiation and longevity of patulin biosynthesis. Dev Industr Microbiol 22: 219–232

Gaucher GM, Wong JM, McCaskill DG (1983) Patulin pathway transformations in *Penicillium urticae* and other fungi. In: Schlessinger D (ed) Microbiology-1983. American Society of Microbiology, Washington, D.C., pp 208–214

GrootWassink JWD, Gaucher GM (1980) *De novo* biosynthesis of secondary metabolism enzymes in homogenous cultures of *Penicillium urticae*. J Bacteriol 141: 443–455

Hahlbrock K (1977) Cell differentiation in micro-organisms, plants and animals. Nover L, Mothes K (eds) Elsevier/North-Holland, pp 524–537

Jones A, Berk D, Lesser BH, Behie LA, Gaucher GM (1983) Continuous production of patulin by immobilized cells of *Penicillium urticae* in a stirred tank reactor. Biotechnol Lett 5: 785–790

Klibanov AM (1983) Immobilized enzymes and cells as practical catalysts. Science 219: 722–727

Lam KS (1981) Ph. D. Thesis. University of Calgary, Calgary, Alberta, Canada

Lynen F, Engser H, Friedrich J, Schindlebeck W, Seyffert R, Wieland F (1978) Fatty acid synthetase of yeast and 6-methylsalicylate synthetase of *Penicillium patulin* – two multienzyme complexes. In: Srere PA, Estabrook RW (eds) Microenvironments and metabolic compartmentation. Academic Press, pp 283–303

Malik VS (1982) Genetics and biochemistry of secondary metabolism. Adv Appl Microbiol 28: 27–115

Marshall R, Field R, Katz F, Weissbach H (1968) Changes in phenoxazinone synthetase during the growth cycle of *Streptomyces antibioticus*. Arch Biochem Biophys 123: 317–323

Martin JF, Liras P (1981) Biosynthetic pathways of secondary metabolism in industrial microorganisms. In: Rehm HJ, Reed G (eds) Biotechnology. Vol 1, Verlag Chemie, pp 211–233

Martin JF, Demain AL (1980) Control of antibiotic biosynthesis. Microbiol Rev 44: 230–251

Neway J, Gaucher GM (1981) Intrinsic limitations on the continued production of the antibiotic patulin by *Penicillium urticae*. Can J Microbiol 27: 206–215

Queener S, Swartz R (1979) Penicillins: Biosynthetic and semisynthetic. In: Rose AH (ed) Economic microbiology. Vol. 3, Academic Press, pp 35–121

Vanek Z, Mikulik K (1978) Microbiol growth and production of antibiotics. Folia Microbiol 23: 309–328

Yamamoto LA, Segel IH (1966) The inorganic sulfate transport system of *Penicillium chyrsogenum*. Arch Biochem Biophys 114: 523–538

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