Arch Microbiol (1994) 162:114–119

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ORIGINAL PAPER

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Leucine interference in the production of water-soluble red *Monascus* pigments

Received: 7 September 1993 / Accepted: 8 February 1994

Abstract The formation of soluble *Monascus* red pigments is strongly positively and negatively regulated by different amino acids. Leucine, valine, lysine, and methionine had strong negative effects on pigment formation. Leucine supported poor pigment formation when used as sole nitrogen source in fermentations, yet it neither repressed pigment synthase(s) nor inhibited its action. The new pigments derived from the hydrophobic leucine were more hydrophilic than the conventional red pigments (lacking an amino acid side-chain) and were extracellularly produced. Therefore, the low level of red pigments produced when leucine was the nitrogen source was not due to feedback regulation by cell-bound leucine pigments. The negative effect of leucine was caused by enhanced decay of pigment synthase(s). The enhanced decay was not due simply to de novo synthesis of a leucine-induced protease.

Key words Pigments · *Monascus* pigments · Leucine · Amino acids · Secondary metabolism

Abbreviations *mSG* Monosodium glutamate
MOPS 3-(*N*-morpholine)propane sulfonic acid
DCW dry cell weight

Introduction

Species of *Monascus* fungus have been known to Westerners as contaminants of cereals, starch, silage, and other materials for 100 years; however, the organism has found many uses in the Orient. The color of Chinese red rice (Ang-Khak or An-Ka) is produced by the growth of *Monascus* spp. on rice. Pigments produced by *Monascus* spp. have been used as a natural food colorant for fish, bean curd, and wine. The fungus has been utilized as an enzymatic agent to make Chinese fermented food, and in folk medicine in the Orient for hundreds of years. Since many coal tar dyes are carcinogenic, the natural pigments produced by *Monascus* spp. are being favorably looked upon as safe natural food colors and replacements for synthetic pigments.

Monascus spp. are well known for their production of six related water-insoluble "conventional" pigments (Fig. 1 A) (Sweeny et al. 1981). Based on observations using isotopes, *Monascus* pigments are probably made by polyketide-forming enzymes (Birch et al. 1962; Whalley 1963; Chen et al. 1971; Manchand 1973). We previously reported that new, water-soluble red pigments are produced by growing and resting cells of *Monascus* sp. incubated in the presence of an amino acid provided as the nitrogen source (Fig. 1 B) (Lin et al. 1992). The new pigments appear to be produced by the polyketide pathway (Lin et al. 1992; Lin and Demain 1993), followed by attachment of the amino acid side-chain. Conflicting conclusions in the literature about nutrient effects on *Monascus* pigment production are due to the use of complex media and different strains of *Monascus* (see Lin 1991 for review).

We recently studied the effect of nutrition on the formation of new pigments by growing and resting cells (Lin and Demain 1991, 1993) and observed strong regulation by amino acids as nitrogen sources. Amino acids act as side-chain precursors for the production of new soluble red pigments. Glutamate is the best sole nitrogen source for pigment production in growth media, but glycine supports higher resting-cell production than glutamate. The resting-cell- and growing-cell systems are useful tools for studying the regulation of complex biosynthetic pathways over and above the effect of nutrients as precursors or energy sources (Demain and Kennel 1978; Hu et al. 1984). In this study, we attempt to determine whether the effects of amino acids are exerted at the level of formation, action, or stability of pigment-forming enzymes (pigment synthases).

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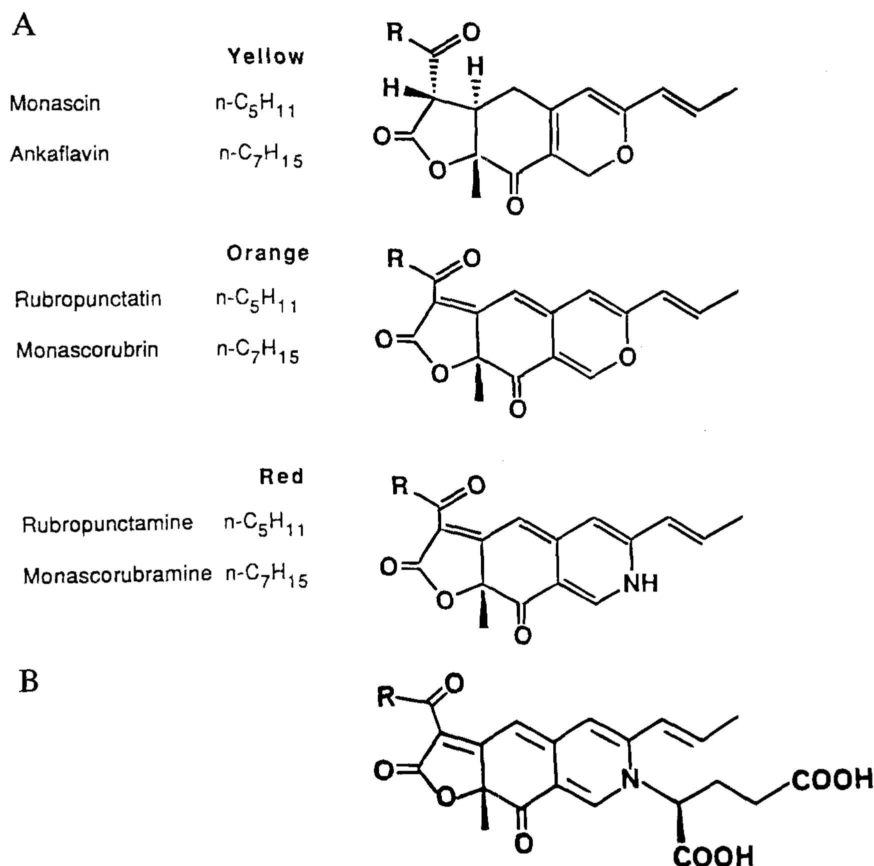

Fig. 1 A, B Structures of *Monascus* pigments. **A** Conventional pigments; **B** red water-soluble glutamate derivatives of the conventional orange pigments. Figures from Lin (1991)

Materials and methods

Microorganisms and cultivation methods

Monascus sp strain TTWMB 6093 was used. This strain produces large amounts of pigment and was obtained by screening (Lin and Huang 1983).

The chemically defined medium previously reported (Lin et al. 1992) was used as the fermentation medium. It contained per l of distilled water: maltose, 50 g; monosodium glutamate (MSG), 12.6 g; K_2HPO_4, 2.4 g; KH_2PO_4, 2.4 g; $MgSO_4 \times 7H_2O$, 1.0 g; KCl, 0.5 g; $ZnSO_4 \times 7H_2O$, 10 mg; $FeSO_4 \times 7H_2O$, 10 mg; $MnSO_4 \times H_2O$, 3 mg. The initial pH of the medium was 5.5.

The stock culture was maintained on YM agar containing: yeast extract, 3 g; malt extract, 3 g; peptone, 5 g; glucose, 20 g; agar, 15 g; and distilled water, 1 l. Mycelial blocks were used for preparing seed cultures. The inoculated seed cultures were grown in YM broth (which contains the same nutrients as YM agar, but lacks agar) at 30° C and 250 rpm for 3 days. Then, a 10% volume of seed culture was inoculated into fermentation medium and incubated at 30° C and 250 rpm for 6 days or other specified times.

Chemicals

The six authentic conventional pigments produced by *Monascus* were supplied by Dr. J. G. Sweeny of the Coca-Cola Company, Atlanta, Ga. Other chemicals were pruchased commercially and were of the highest purity available, the major source being Sigma Chemical Company (St. Louis, Mo. USA).

Preparation of resting-cell incubation system.

After 36 h (or other indicated times) of incubation, the fermentation broth was filtered. The resting-cell incubation system was prepared as previously described (Lin et al. 1992).

Estimation of fermentative and resting-cell production of pigments

At the end of the fermentation or resting-cell incubation, pigment productivity was measured as previously described (Lin et al. 1992).

Dry cell weight (DCW)

After extraction of cell-bound pigment, the mycelia were dried at 80° C for 24 h and then weighed.

Effects of added compounds

For the study of regulatory effects, various nitrogen sources, with or without the protein-synthesis inhibitor cycloheximide, were added to the resting-cell incubation mixture or fermentation medium for examination of their positive or negative effects in comparison with controls.

Variation

Variation in duplicate or triplicate samples in any one experiment was less than 10%. Between individual experiments, variation was 20% or less.

Results and discussion

Effect of nitrogen source on pigment production by growing cells

To investigate whether nitrogen sources exert any regulatory function in addition to serving as nitrogen sources for growth and as side-chain precursors of red pigments (Lin et al. 1992), NH_4NO_3 and 20 amino acids were first compared as sole nitrogen sources at concentrations supplying 75 m*M* nitrogen in the presence of 150 m*M* 3-(*N*-morpholine)propane sulfonic acid (MOPS) buffer. MSG, with and without MOPS buffer, was used as a nitrogen source control. All media were adjusted to an initial pH of 6.8, except the "MSG without MOPS" control, which had an initial pH of 5.5.

As expected from our earlier experiments (Lin and Demain 1991), MSG supported much more pigment production than NH_4NO_3 (Table 1); histidine was almost as active. All nitrogen sources supported growth. Although some supported better growth than MSG, none approached MSG in its ability to support red pigment production. MOPS (150 m*M*) failed to stabilize the pH in some cases. Low pH values were especially observed with glycine and alanine; moderate pH reduction was observed with glutamine, methionine, isoleucine, and leucine.

Table 1 Effect of different nitrogen sources on growth, pH, and pigment formation during fermentation in the presence of 150 m*M* MOPS

Sole nitrogen source	Final pH	DCW[a] (mg/ml)	Total OD_{500}	Specific OD_{500}[b]
MSG (no MOPS)	6.8	9.24	74.6	8.07
MSG	7.0	7.84	77.6	9.90
His	6.6	5.69	51.6	9.07
Ser	6.4	10.90	57.9	5.31
Arg	6.6	10.50	49.3	4.69
Phe	6.3	9.18	41.4	4.51
Asn	6.5	11.10	35.0	3.15
Ile	5.5	11.80	37.1	3.14
Thr	6.7	8.23	24.6	2.99
Asp	6.7	8.03	23.4	2.91
Tyr	6.5	12.40	34.2	2.76
Ala	3.6	9.51	24.8	2.60
Pro	6.6	8.45	21.6	2.56
Leu	5.8	11.40	28.0	2.46
Cys	6.6	11.80	27.9	2.36
Gly	4.1	13.60	27.7	2.04
Lys	6.6	7.57	14.5	1.92
Gln	5.8	12.80	21.9	1.71
NH_4NO_3	6.7	8.32	12.0	1.44
Val	6.5	8.67	12.1	1.40
Met	5.5	–[c]	10.1	–[c]
Trp	6.3	–[c]	8.9	–[c]

[a] Dry cell weight
[b] Total OD_{500}/DCW
[c] Met and Trp did not completely dissolve during the 6 day fermentation

Some of the amino acids that produced low pH values and five amino acids (MSG, histidine, serine, arginine, phenylalanine) that led to the best pigment production (Table 1) were compared in the presence of 200 m*M* MOPS buffer. The pH of the fermentation media was checked every day after the third day of fermentation, and the pH was maintained at 6.6 by adding 0.1 *N* KOH. In the presence of 200 m*M* MOPS buffer, the five amino acids that previously led to low pH values, and also serine, required pH adjustment on the fourth and fifth day of fermentation. Although the addition of KOH stabilized their pH values close to 6.6, the specific pigment production supported by these five amino acids was only slightly better than that observed in 150 m*M* MOPS buffer without addition of KOH during fermentation. Again, MSG and histidine, led to the best pigment formation by growing cells. Although histidine supported high specific pigment production, total pigment formation and growth were low, making it a less desirable nitrogen source for fermentations than MSG. Since many amino acids are excellent nitrogen sources for growth and since OD_{500} measures the basic pigment structure irrespective of the presence or absence of an amino acid side-chain (see next section), the above data indicate that amino acids have effects other than provision of nitrogen for growth or as side-chain precursors for pigment molecules.

Negative effects of nitrogen sources in the presence of glutamate

In order to examine whether NH_4^+, NO_3^- and leucine (poor sole nitrogen sources for pigment production) have negative effects on pigment production in the presence of MSG, the initial pH was kept at 6.8 and 200 m*M* MOPS buffer was used. The results are shown in Table 2. Within the range of 37.5 to 75 m*M*, an increase of MSG concentration caused no marked changes in growth or pigment production. In the presence of MSG, leucine caused a significant increase in growth and a very dramatic drop (ca. 80%) in pigment production. Neither KNO_3 nor NH_4Cl affected total pigment formation, but the latter stimulated growth, causing a decrease in specific pigment production.

Nineteen amino acids, (37.5 m*M*) were next examined to determine the degree to which each influences pigment production in the presence of 37.5 m*M* K-glutamate and 200 m*M* MOPS buffer at an initial pH of 6.8. The interfering amino acids and their degree of interference of specific pigment formation were found to be as follows: valine (85%), lysine (81%), leucine (78%), tryptophan (57%), tyrosine (56%), histidine (53%), and isoleucine (38%). Methionine could not be evaluated in the same manner because its failure to dissolve completely made optical measurement of growth difficult. However, it did interfere in total pigment formation by 77%. Phenylalanine and cysteine interfered to a minor extent and the remaining amino acids showed no negative effect.

Table 2 Effect of KNO_3, NH_4Cl and leucine on growth, pH, and pigment formation during fermentation in the presence of MSG and 200 m*M* MOPS

Nitrogen source (mM)	Final pH	DCW[a] (mg/ml)	Total OD_{500}	Specific OD_{500}[b]
MSG (37.5)	6.9	7.60	68.9	9.07
MSG (56.2)	6.9	7.81	73.3	9.39
MSG (75.0)	6.9	7.92	77.7	9.81
MSG (37.5) + KNO_3 (37.5)	7.0	7.17	70.9	9.89
MSG (56.2) + KNO_3 (18.8)	7.0	7.03	69.4	9.87
MSG (37.5) + NH_4Cl (37.5)	7.0	13.30	77.4	5.82
MSG (56.2) + NH_4Cl (18.8)	6.9	12.20	77.8	6.38
MSG (37.5) + Leu (37.5)	6.8	11.30	19.2	1.70
MSG (56.2) + Leu (18.8)	6.9	11.10	17.7	1.61

[a] Dry cell weight
[b] Total OD_{500}/DCW

Since different amino acid derivatives of pigments may be produced from these different amino acids, a true comparison of pigment production can only be made after obtaining information on the distribution of these different pigment derivatives, and their individual absorption coefficients. HPLC analysis showed that pigments produced in medium containing both glutamate and leucine are mainly glutamate derivatives, accompanied by a small amount of leucine pigments. Furthermore, the absorption coefficients of three different amino acid derivatives (glutamate, glycine, and leucine) were found to be quite similar (data not shown). Based on the assumption that the absorption coefficients of different amino acid derivatives are similar, we conclude that a number of amino acids interfere with pigment formation and that leucine is one of the most active.

Enhanced decay of pigment productivity induced by leucine

The above fermentation experiments showed that leucine as sole nitrogen source supports very poor pigment production (Table 1) and, when added to glutamate, depresses pigment production (Table 2). However, previous resting-cell data had shown that leucine supported very good production, similar to that by MSG (Lin et al. 1993). The pigments derived from the hydrophobic leucine are more hydrophilic than the conventional red pigments and are extracellularly produced. Another resting-cell experiment in the presence of leucine was conducted for 24 h instead of the usual 1.5 h; glycine was added as positive control. Leucine again stimulated resting-cell pigment production as did glycine. HPLC analysis indicated that resting cells in the presence of leucine produce red pigments with a leucine side chain (retention time 13.0 and 14.2 min). Since leucine had the same stimulatory effect as glycine (one of the best amino acids for pigment production by resting cells), it is obvious that leucine does not inhibit the action of pigment-forming enzymes.

A possible repressive or inactivating effect of leucine on pigment synthase(s) in fermentation media was studied by comparing resting-cell pigment productivity between glutamate-grown and leucine-grown populations of dif-

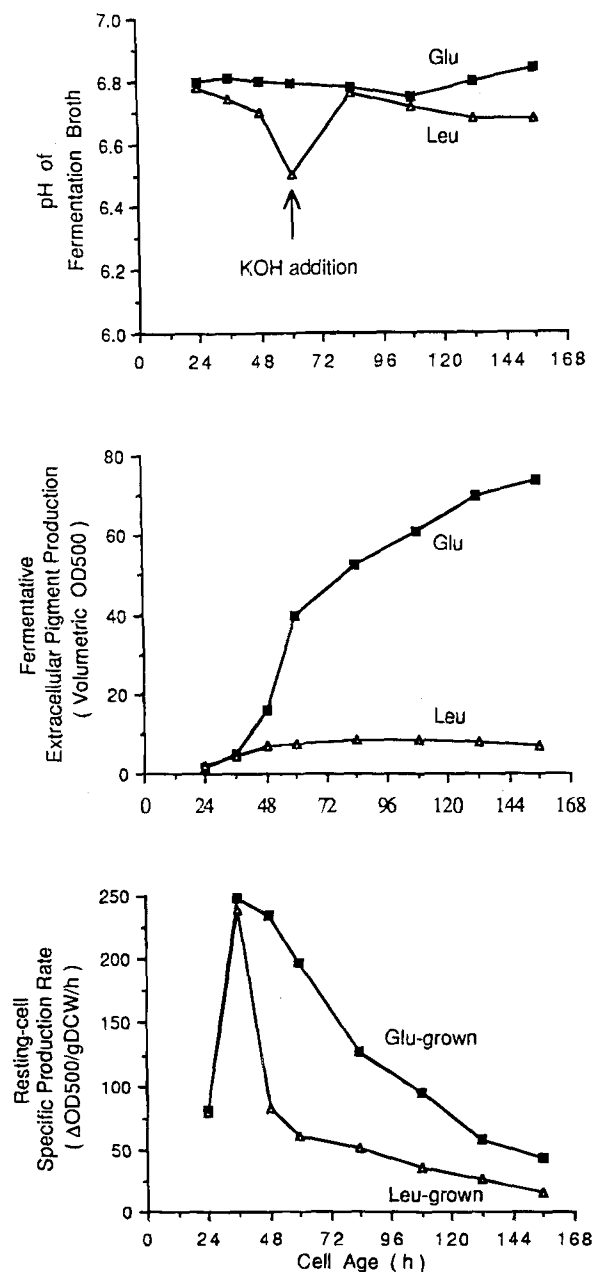

Fig. 2 Effect of time on extracellular pigment production in fermentation medium containing glutamate or leucine and effect of age of cell population on resting-cell pigment productivity

ferent ages. The two fermentations conducted to prepare the cells are shown in Fig. 2. The middle section shows the poor pigment production in the fermentation containing leucine. It can also be seen that resting-cell pigment productivity (of extracellular pigments) was the same for glutamate and leucine when 24- and 36 h populations were used. It is obvious that there is no repressive effect of leucine on enzymes of the pigment pathway. If there had been repression, the productivity of the leucine-grown cells would have been lower than that of the glutamate-grown cells. The peak of specific resting-cell pigment production during growth on either glutamate or leucine occurred after 36 h. After this peak, resting cells grown in leucine lost productivity much more rapidly than cells grown in glutamate. Leucine-grown populations at 48 h had only 35% of the activity of leucine grown populations at 36 h, but glutamate-grown populations at 48 h still retained 94% activity. The rapid drop in productivity of leucine-grown cells, probably due to enzyme inactivation, resulted in a virtual cessation of extracellular pigment production in leucine fermentation broths after 48 h (middle section, Fig. 2). On the other hand, the extracellular pigment accumulation in glutamate medium continuously increased up to 156 h. The uninterrupted accumulation of pigment in the glutamate fermentation medium while specific resting-cell production was decreasing is due to a continuous increase of cell mass in the glutamate fermentation medium (Lin and Demain 1991). It should be noted, however, that the rate of fermentative pigment formation dropped as the resting-cell specific productivity took its marked drop at 60 h.

The pH values of the glutamate medium remained at the initial value of 6.8 throughout the 6-day fermentation, but the pH values of the leucine medium decreased to 6.5 at 60 h. After the addition of KOH to bring the pH back to 6.8, the pH value of the leucine medium dropped only slightly. After 6 days of fermentation, there was no detectable sugar left in either the glutamate or leucine fermentation broth. Thus, there is probably more acid produced in the leucine medium than in the presence of glutamate.

The lack of a negative effect of leucine on resting-cells even in a 24-h incubation, but a negative effect on growing-cells (Fig. 2) could be due to two reasons. First, de novo synthesis of some protein, e.g., a protease, might be required for the rapid decay of pigment synthase(s), and this could not occur in the presence of cycloheximide, a component of the resting-cell system. Second, toxic levels of organic acids may accumulate in fermentation broth after 36 h, but may not during the 24-h resting-cell incubation. These two possibilities are discussed in the next two sections.

Table 3 Effect of cycloheximide addition on fermentative pigment production by cells growing in leucine

Amino acid for growth	Time of cyclo-heximide addition	DCW (mg/ml)	Specific production (total OD_{500}/DCW)	Final pH
Glu (control)	None	3.94	9.53	6.72
Leu	36 h	2.33	3.30	6.67
	48 h	5.82	2.12	6.59
	None	6.97	1.53	6.49

Fermentations were analyzed at 60 h of incubation

Effect of cycloheximide

Polyketide-forming enzymes are known to have rather limited stabilities in vivo (Neway and Gaucher 1981). In certain cases, such instability is caused by intracellular proteases (Bond and Butler 1987). One possible reason for the occurrence of leucine enhancement of synthase decay in fermentations, but not in the resting-cell system, is that cycloheximide in the resting-cell system is masking a potentially negative effect of leucine, e.g., cycloheximide might inhibit the formation of a leucine-induced protease that destroys pigment synthase(s). Thus, the omission of cycloheximide from resting-cell incubation mixtures was examined with respect to its effect on pigment production. This omission did not decrease pigment production in the presence of leucine or glycine as amino acid source. Instead, the omission of cylcoheximide slightly increased both extracellular and cell-bound pigment production. Thus, allowing de novo synthesis of proteins in glutamate-grown cells harvested at 36 h of fermentation had no negative effect on pigment production, indicating that the much better performance of leucine in resting cells than in growing cells is not due to an inhibition of protease formation in the cycloheximide-containing resting-cell system.

We also carried out the reverse experiment, i.e., adding cycloheximide to growing cells early in the fermentation. Cycloheximide (100 μg/ml) was added to cells growing in leucine at 36 or 48 h in the fermentation and the fermentation broth was harvested at 60 h. The results are summarized in Table 3. This addition of cycloheximide inhibited growth and showed a mild improvement in specific production of pigments. Addition of cycloheximide at 36 h was better than addition at 48 h. However, the production value did not approach that occurring with glutamate. Thus, de novo synthesis of protein (protease?) after 36 h of fermentation is not sufficient to explain the enhanced decay of pigment synthase(s) in cells growing with leucine.

To study the possibility that an earlier addition of cycloheximide might show a greater improvement of leucine performance in the fermentation, the effect of early addition (28 h vs 36 h) to cells growing in leucine was examined. In this experiment, resting-cell productivity of such cells was determined at 60 h of fermentation. We also added cycloheximide to cells growing in glutamate. The addition of cycloheximide at 28- or 36 h to cells growing in leucine or glutamate caused only minor effects on pigment synthase stability. Again, the data do not support a mechanism involving leucine-enhanced pro-

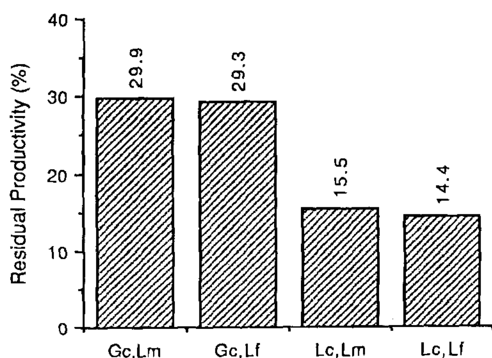

Fig. 3 Comparison of a spent leucine filtrate *(Lf)* with a fresh leucine medium *(Lm)* on stability of pigment synthases during a secondary fermentation (24 h) of a 36 h leucine-grown population *(Lc)* and a 36 h glutamate-grown population *(Gc)*. Residual productivity is calculated by comparison to productivity of 36 h populations, which is taken as 100%

tease formation as the reason for the more rapid loss of pigment synthase(s) in leucine fermentations.

Leucine vs leucine metabolite(s) as effector of synthase lability

In order to examine whether the rapid disappearance of pigment synthase(s) is due to leucine itself or to leucine metabolites (e.g., acids) accumulated in the fermentation broth, we filtered the mycelia from leucine medium and glutamate medium fermentations at 36 h. The two sets of mycelia were then distributed into two different media, the spent filtrate of the 36-h fermentation broth from leucine-containing medium and a fresh leucine medium, at a final cell concentration (wet weight) of 3 g/40 ml broth. The pH value of the two media was adjusted to 6.8 before the secondary fermentation. After a secondary fermentation of 24 h, the mycelia in the two different media were harvested by centrifugation and washed twice with MOPS-cycloheximide buffer as in the usual resting-cell procedure. The resting-cell pigment productivities of the four different types of mycelia were then compared.

As expected, leucine-grown cells lost their resting-cell productivity faster than glutamate-grown cells during the secondary fermentation in both media. There was almost no difference in resting-cell productivity between the spent leucine filtrate and fresh leucine medium (Fig. 3). This result indicates that metabolites, which might accumulate in leucine medium during 36 h of fermentation, are no more active than leucine itself in accelerating the decay of pigment synthase(s).

The overall conclusion from the studies of leucine regulation is that leucine acts by enhancing the decay of pigment synthase(s). The enhanced decay cannot be explained simply be de novo synthesis of a leucine-induced protease. If such a protease plays a role, it is a minor one. The major mechanism of the leucine-induced instability of the enzyme(s) remains to be elucidated.

Acknowledgements T. F. Lin was partially supported by the Taiwan Provincial Government and the Taiwan Tobacco and Wine Monopoly Bureau, Republic of China. We thank J. G. Sweeny of the Coca-Cola Company (Atlanta, Ga, USA) for gifts of the six authentic *Monascus* pigments. We also acknowledge the following companies for their general financial support of students in this laboratory: American Cyanamid Co., Amgen Inc., Amoco Technology Corp., Aspen Technology Inc., Biochemie GmbH, Bioindustrie Mantovane Sri, Biopure Corp., Bristol Myers-Squibb Inc., Centocor Inc., Ciba-Geigy Corp., DSM Corp., Fujisawa Pharamceutical Co., G. D. Searle & Co., Hoffmann-La Roche Inc., International Flavors & Fragrances Inc., Kanebo Ltd., Kyowa Hakko Kogyo Co. Ltd., Merck & Co. Inc., Monsanto Agricultural Products Co., Mycosearch Inc., Nippon Kayaku Co. Ltd., Schering-Plough Research, Stroh Brewery Co., Sumitomo Chemical Co. Ltd., Sygena Ltd., Toyo Engineering Corp., Universal Foods Corp., and Wyeth-Ayerst Research.

References

Birch AJ, Cassera A, Fitton P, Holker JS, Smith H, Thompson GA, Walley WB (1962) Studies in relation to biosynthesis, part 30. Rotiorin, monascin, and rubropunctatin. J Chem Soc 3583–3586

Bond JS, Butler PE (1987) Intracellular proteases. Annu Rev Biochem 56: 333–364

Chen FC, Manchand PS, Whalley WB (1971) The chemistry of fungi, part 54. The structure of monascin: the relative stereochemistry of the azaphilones. J Chem Soc (C) Org Chem 3577–3579

Demain AL, Kennel YM (1978) Resting-cell studies on carbon source regulation of β-lactam antibiotic biosynthesis. J Ferment Technol 56: 323–328

Hu WS, Braña AF, Demain AL (1984) Carbon source regulation of cephem antibiotic production by resting cells of *Streptomyces clavuligerus* and its reversal by protein synthesis inhibitors. Enzyme Microbiol Technol 6: 155–160

Lin TF (1991) Studies on the formation of *Monascus* red pigments. Ph D Thesis, Massachusetts Institute of Technology, Cambridge, Mass., USA

Lin TF, Demain AL (1991) Effect of nutrition on formation of *Monascus* red pigments. Appl Microbiol Biotechnol 36: 70–75

Lin TF, Demain AL (1993) Resting cell studies on formation of water-soluble red pigments by *Monascus* sp. J Ind Microbiol 12: 361–367

Lin TF, Huang CT (1983) The zymotic properties of *Monascus* mold. The production of extracellular amylases (in Chinese). Ann Report Res Inst Wines 157–167

Lin TF, Yakusijin K, Büchi G, Demain AL (1992) Formation of water-soluble *Monascus* red pigments by biological and semisynthetic processes. J Indust Microb 9: 173–179

Manchand PS, Whalley WB (1973) Isolation and structure of ankaflavin: a new pigment from *Monascus anka*. Phytochemistry 12: 2531–2532

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

Sweeny JG, Estrada-Valdes MC, Iacobucci GA, Sato H, Sakamura S (1981) Photoprotection of the red pigments of *Monascus anka* in aqueous media by 1,4,6-trihydroxynaphthalene. J Agric Food Chem 29: 1189–1193

Whalley WB (1963) The sclerotiorin group of fungal metabolites: their structure and biosynthesis. Pure Appl Chem 7: 565–587