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Studies on the Overproduction of Indole-Containing Metabolites by a Methanol-Utilizing Yeast, *Hansenula polymorpha*

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Abstract

Production of indole-containing metabolites ("indoles") from methanol has been studied using a mutant of *Hansenula polymorpha* resistant to 5-fluorotryptophan. Whereas the wild-type culture produces only a small amount of indoles, the mutant is partially deregulated and overproduces indoles. Indoles production was studied in batch and continuous culture and in a washed-cell system. When the pH was above 4.0, indoles production was growth-associated, in both minimal and complex media, and batch or continuous culture. When the pH was below or equal to 4.0, a low phosphate concentration was found to improve production. In a phosphate-deficient washed-cell suspension system, the addition of an amino acid such as methionine at 5 m*M* increased specific productivity by more than 60%. Addition of cycloheximide at 50 mg/L decreased residual growth and increased maximum productivity of indoles by more than 60%. When the antibiotic was added at 1000 mg/L, growth was completely inhibited and indoles production continued for about 35 h.

Index Entries: Methanol utilization, by the yeast, *Hansenula polymorpha;* indoles, overproduction, by a methanol utilizing yeast; fermentation, of a yeast for indoles production; metabolites, yeast produced indole-containing; yeast, production of indoles by; *Hansenula polymorpha,* production of indoles from.

Introduction

The objective of this work was to environmentally examine indoles production by the methanol-utilizing yeast, *Hansenula polymorpha*. Methanol has received at-

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tention as a potential carbon-energy source for fermentations because of its relatively low cost and attractive biotechnological features (e.g., miscibility with water, reduced risks of contamination) (*3, 9, 11*).

An indoles-producing mutant isolated by Denenu and Demain (*5*) from the methanol-consuming yeast, *Hansenula polymorpha* (*8*), was used in this work. We used different culture conditions—batch, continuous culture, and a washed-cell system (WCS)—to study the effect of the environment on indoles overproduction.

Materials and Methods

Organisms

The wild-type strain *H. polymorpha* DL-1 (ATCC 26012) (*8*) and a mutant strain (3-136) resistant to 5-fluorotryptophan (*5*) were used in this study.

Media

The minimal medium used in batch or continuous culture experiments contained the following compounds, in g/L: $(NH_4)_2SO_4$, 5.0; $MgSO_4 \cdot 7H_2O$, 1.15; $CaCl_2 \cdot 2H_2O$, 0.1; NaCl, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.0014; $MnSO_4 \cdot H_2O$, 0.00084; $FeSO_4 \cdot 7H_2O$, 0.00028; $CuSO_4 \cdot 5H_2O$, 0.00025; $Na_2MoO_4 \cdot 2H_2O$, 0.00024; $CoCl_2 \cdot 6H_2O$, 0.00024; thiamine, 0.0004; biotin, 0.000002; and various amounts of KH_2PO_4 and methanol, as described below. Methanol and vitamins were added aseptically to the rest of the medium after the latter was adjusted to pH 5.5 (with NaOH) and autoclaved. Complex medium contained 0.5 g yeast extract/L of the above. The WCS medium (which was devoid of phosphate) contained the following in g/L: $(NH_4)_2SO_4$, 5.0; $MgSO_4 \cdot 7H_2O$, 1.15; $CaCl_2 \cdot 2H_2O$, 0.1; NaCl, 0.1; KCl, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.0014; $MnSO_4 \cdot H_2O$, 0.00084; $FeSO_4 \cdot 7H_2O$, 0.00028; $CuSO_4 \cdot 5H_2O$, 0.00025; $Na_2MoO_4 \cdot 2H_2O$, 0.00024; $CoCl_2 \cdot 6H_2O$, 0.00024; thiamine, 0.0004; biotin, 0.000002; *N*-2-hydroxyethylpiperazine, *N'*-3-propanesulfonic acid (HEPPS buffer) 31.5 (125 m*M* final concentration). The initial pH was adjusted to 7.45. In one experiment, instead of HEPPS buffer, we used 2-(*N*-morpholino)-ethane-sulfonic acid sodium salt (MES buffer). Buffers and, when needed, methanol, amino acids, and antibiotics, were added aseptically to the rest of the medium.

Vitamins, amino acids, and antibiotics were sterilized by filtration. The remaining components were autoclaved at 120°C for 20 min.

Batch and WCS Cultures

Fifty-milliliter aliquots of medium in 250-mL Erlenmeyer flasks were inoculated with an exponential phase culture. The flasks were incubated at 37°C on a rotary shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 220 rpm. For the WCS experiments, log-phase cells produced in batch cultures containing 66 m*M* phosphate and 10 g/L methanol were centrifuged, washed twice in two-fold con-

centrated salts solution of the WCS medium, and resuspended to achieve a dry cell weight (DCW) concentration of 2-2.5 g/L in the phospate-free WCS medium. The initial methanol concentration was 20 g/L. Cultures were grown in 15-cm tubes (internal diameter, 1.5 cm) containing 2.5 mL of the cell suspension under the same conditions as the flask cultures.

Continuous Culture Apparatus

The 1-L fermentor containing 350 mL of medium was as previously described (*2*).

Growth Measurements

Growth was estimated by absorbance in a Klett-Summerson colorimeter with a red filter (No. 66). Two hundred sixty Klett units (KU) are equivalent to 1 g DCW/L. All values were corrected for evaporation.

Methanol Determination

Methanol was measured in a Varian Aerograph 1200 gas chromatography apparatus (*8*).

Colorimetric Determination of Indoles

The xanthydrol assay of Dickman and Crockett (*7*) was used to estimate indoles in the culture supernatant. A Beckman DB-GT spectrophotometer was used for absorbance measurements at 510 nm. Concentrations were estimated relative to that of a standard L-tryptophan solution.

Chemicals

HEPPS and MES buffers were purchased from Calbiochem and xanthydrol from Eastman Organic Chemicals.

Results

Comparison of Wild-Type and Mutant

In both batch and chemostat cultures, the wild-type strain produced less than 10% of the concentration of indoles produced by mutant 3-136. The following results were obtained with this mutant.

Production in Batch Culture

Effect of Methanol Concentration. The minimal medium containing 66 m*M* phosphate was supplemented with different amounts of methanol from 4 to 24 g/L. The log-phase inoculum was developed in this medium containing 4 g/L methanol. The initial cell density was 1.5 mg DCW/L. Figure 1 shows that the growth rate in

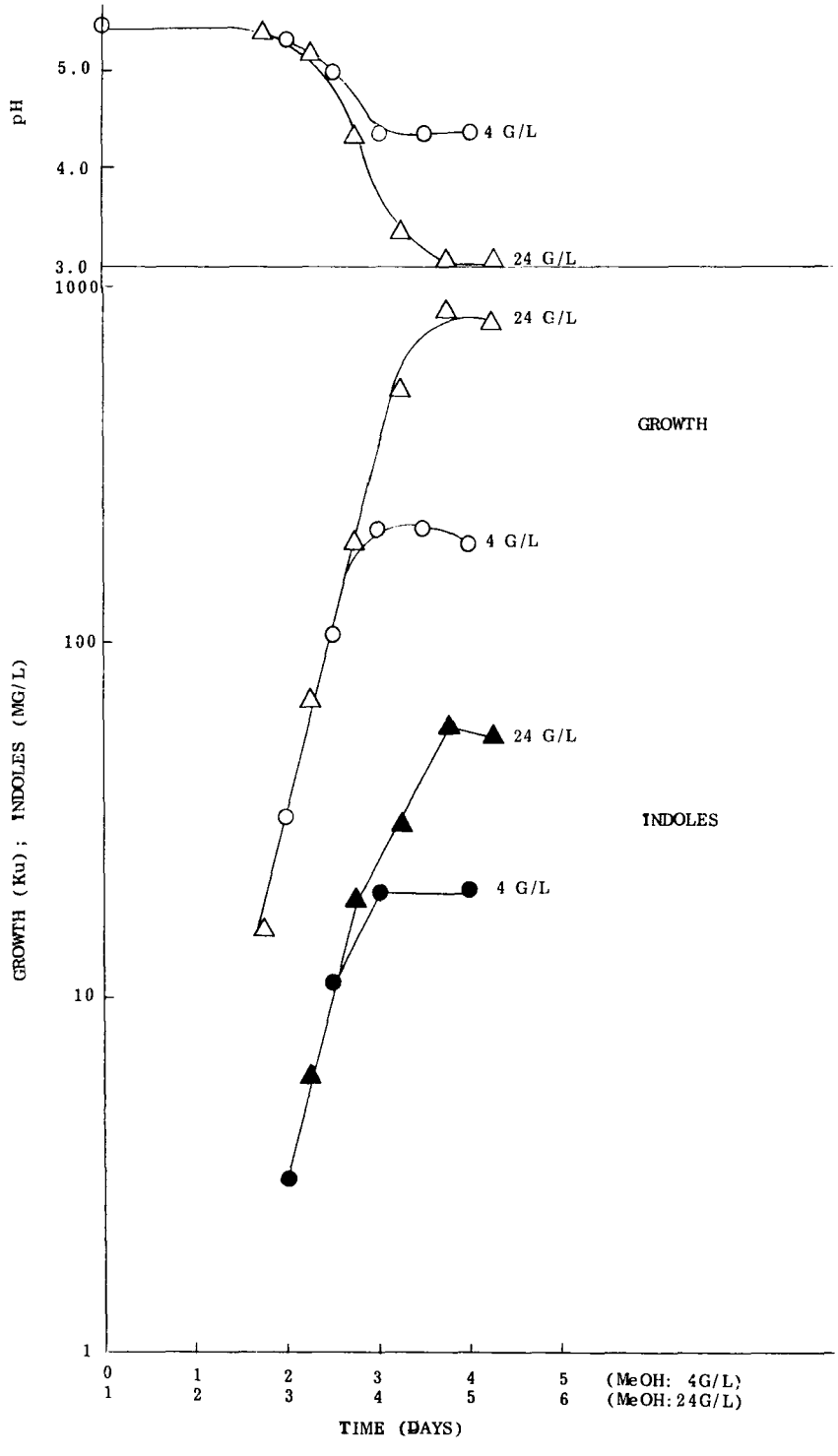

Fig. 1. Growth and indoles production in batch cultures with 66 m*M* phosphate and 4 or 24 g/L methanol.

the culture with 24 g/L methanol was the same as with 4 g/L methanol, although a 24-h lag occurred in the presence of the higher methanol concentration. Indoles production in both cases was growth-associated. The maximum specific productivities in the two media were the same (2.2 mg/g · h) as were the maximum specific production values (27–29 mg/g DCW). In the culture with 24 g/L methanol, the drop in pH to 4.0 and below did not affect the growth rate, but the rate of indoles production decreased.

In Fig. 2, we can see that the final cell concentration increased with increasing initial methanol concentrations initially with a yield of 0.18 g DCW/g methanol fed, above 15 g methanol/L, the cell yield fell to 0.08. The final concentration of indoles increased linearly with methanol until it reached a plateau when methanol reached 15 g/L.

Effect of Phosphate Concentration. The minimal medium containing 24 g/L methanol was supplemented with different amounts of phosphate (66, 6.6, or 0.4 m*M*). Figure 3 shows that the growth level attained was limited by 0.4 m*M* phosphate, but that phosphate was not limiting at 6.6 or 66 m*M*. This limitation corresponds to a cell yield on phosphate of 33 g DCW/g phosphate, a reasonable value.

Concerning indoles production (Fig. 3, Table 1), when the phosphate concentration was high (66 m*M*), specific productivity was very dependent on pH, i.e., it dropped from 2.2 mg/g · h at pH 4.8 to 0.8 mg/g · h at pH 3.3 despite a constant growth rate of 0.075 h^{-1}. Indoles production was 56 mg/L and 17 mg/g DCW. When phosphate concentration was intermediate (6.6 m*M*), increased indoles production was observed (110 mg/L and 38 mg/g DCW). At this phosphate concentration, it appears that the pH drop had a lesser effect on specific productivity, i.e., it only decreased from 2.2 to a value of 1.5 mg/g · h at pH 2.5. At the lowest concentration, indoles production was 73 mg/L and 58 mg/g DCW. Specific productivity remained high (2 mg/g · h) and constant at growth rates between 0.02 and 0.07 h^{-1} and a pH as low as 2.8. At all phosphate concentrations, there was a drastic cessation of indoles production at the end of growth.

Production in the Chemostat

Chemostat studies were undertaken to overcome potential problems of methanol toxicity that generally occur in batch culture with a high initial methanol concentration and to further examine the effect of growth rate on indoles overproduction. These studies were done without pH control, except for the phosphate-limited chemostat.

The results are shown in Table 2. In a methanol-limited chemostat (containing 66 m*M* phosphate), specific productivity was at its maximum (1.25 mg/g · h) at the lowest dilution rate examined, 0.05 h^{-1}; the pH was 4.2. Titers of indoles were 23 mg/L and 25 mg/g DCW. Higher growth rates led to lower indoles production, probably because of the low pH values that developed.

When the methanol-limited chemostat medium included 0.5 g/L yeast extract, indoles production increased with increasing growth rate and all pH values were above 4.0. At a growth rate of 0.15 h^{-1}, productivity reached the highest value

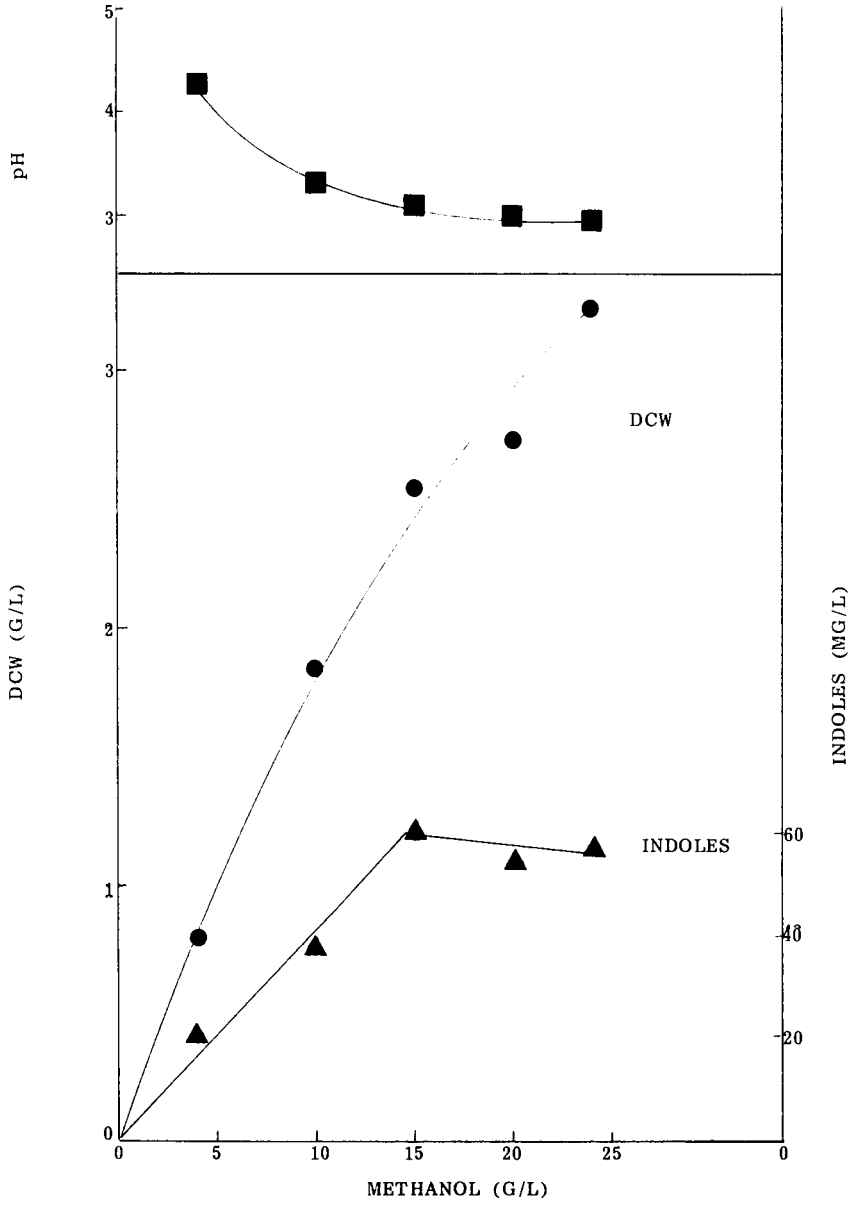

Fig. 2. Final dry cell weight (DCW) and indoles production in batch cultures with 66 m*M* phosphate and with different methanol concentrations.

observed in this study, i.e., 3.5 mg/g · h. The low indoles content in yeast extract was subtracted from all values.

In a phosphate-limited chemostat (dilution rate of 0.045 h^{-1}), in chemically defined medium,the volumetric titer of indoles was much higher than with methanol limitation, i.e., about 100 mg/L, but specific production (24 mg/g DCW) and specific productivity (1.1 mg/g · h) were not higher; pH was 4.0. When the dilution rate was 0.1 h^{-1}, indoles production (mg/L and mg/g DCW) dropped. The rea-

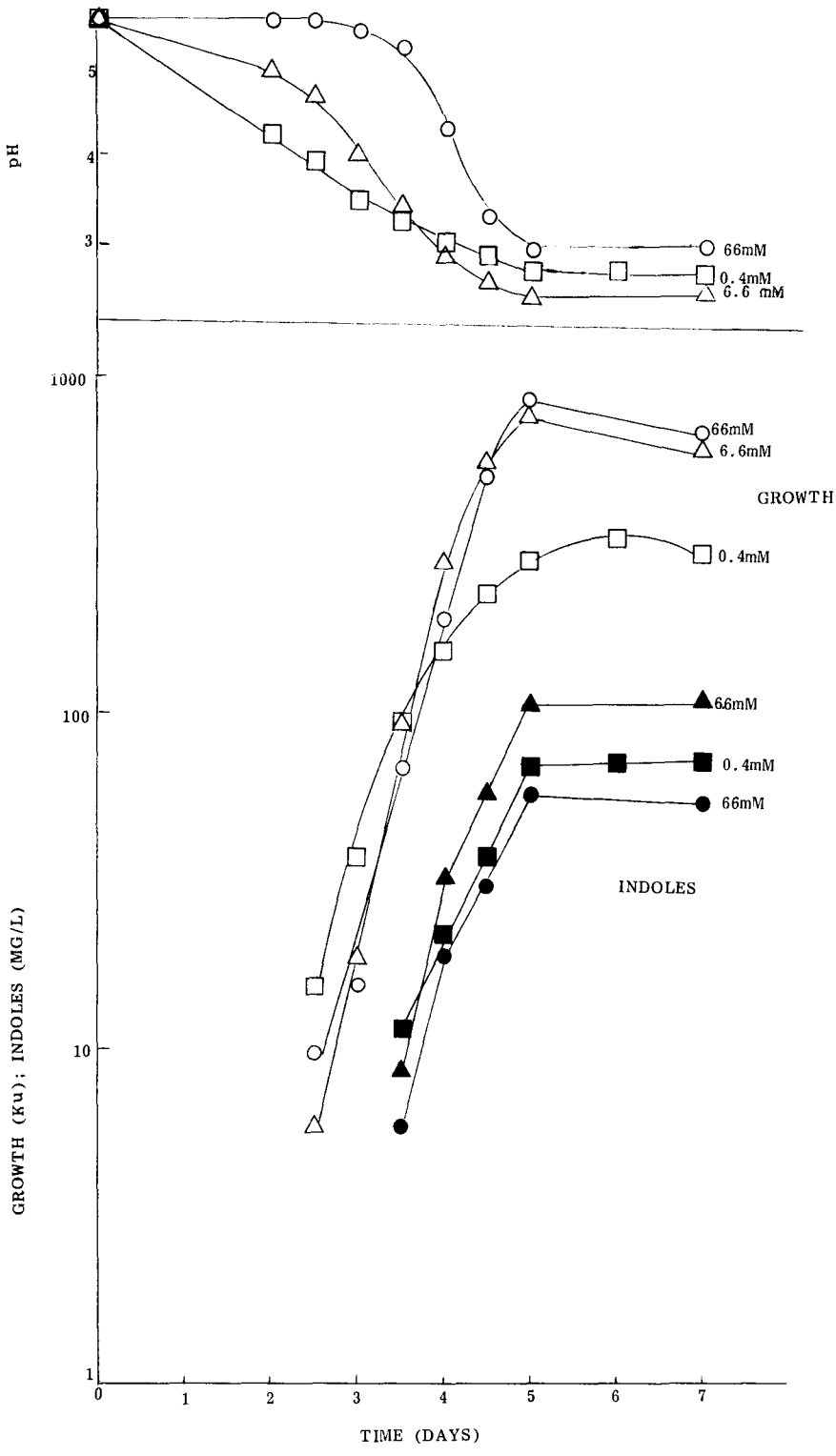

Fig. 3. Growth and indoles production in batch cultures (with 24 g/L methanol) and with different phosphate concentrations.

TABLE 1
Growth and Indoles Production in Batch Culture with 24 g/L Methanol and Different Phosphate Concentrations

Phosphate, m*M*	Final pH	Maximum DCW, g/L	Production of indoles		
			mg/L	mg/g DCW	Maximum productivity[a] mg/g DCW·h
66	2.95	3.2	56	17	2.2
6.6	2.40	2.9	110	38	2.2
0.4	2.65	1.3	73	58	2.2

[a]The maximum productivity was obtained during the early log-phase.

sons are not clear (see Discussion). We should note that there was some foam and cell lysis under these conditions.

Production in a Washed-Cell System

A phosphate-deficient WCS was devised to investigate additional factors regulating indoles overproduction.

Preliminary experiments were undertaken to select a suitable buffer for these experiments. In a 3.6-day experiment with 20 g/L methanol added batchwise, we obtained the results in Table 3. Indoles production was best in the HEPPS buffer, initial pH 7.45, whereas in the MES buffer (initial pH 6.3 or 5.1), growth was better but indoles production was low.

The optimum concentration of the HEPPS buffer was next investigated and found to be 125 m*M* (results not shown). This buffer concentration was used for the rest of this work (initial pH 7.45).

Indoles overproduction using excess methanol (initial concentration of 20 g/L and fed intermittently every 2 days in 20 g/L portions) amounted to 330 mg/L and 80 mg/g DCW in 6 days (Fig. 4). Maximum productivity was 1.5 mg/g · h. The volumetric productivity was linear up to 6 days at 2.3 mg/L · h.

Since methionine has an effect on aromatic amino acid biosynthesis in *Bacillus subtilis* (*10*), we studied the effect of this amino acid in the WCS. Indeed, this compound had a positive effect on indoles production and the optimal concentration in a 2-day preliminary experiment was found to be 5 m*M* (results not shown).

Figure 4 shows the effect of L-methionine. When the concentration of methionine was 5 m*M*, the volumetric rate of indoles production was linear for 3 days and decreased after that time. When methionine was fed intermittently every 2 days, the volumetric production was linear for 6 days. In both cases of methionine supplementation, the maximum specific productivity was increased to 2.5 mg/g · h compared to 1.5 mg/g · h without methionine. With methonine feeding, titers were essentially doubled to 600 mg/L and 158 mg/g DCW.

Twenty amino acids were each added in the WCS at a 5-m*M* final concentration. Methionine showed a 54% increase in specific indoles production in the 1.3-day experiment. Other amino acids that markedly improved production were L-threo-

TABLE 2
Growth and Indoles Production in Continuous Culture

Medium	Limiting nutrient	Dilution rate, h^{-1}	Cell concentration, g DCW/L	Production of indoles			pH (at steady state)[a]
				mg/L	mg/g DCW	mg/g DCW·h	
Minimal medium							
Phosphate: 66 m*M*	Methanol	0.05	0.92	23	25	1.3	4.2
Methanol: 4 g/L		0.10	1.24	11	9	0.9	3.5
pH of feed: 5.5		0.15	1.16	8	7	1.0	3.7
Complex medium							
Phosphate: 66 m*M*	Methanol	0.05	1.21	23	19	1.0	4.9
Methanol: 4 g/L		0.10	1.32	20	16	1.6	4.2
Yeast extract: 0.5 g/L		0.15	1.42	33	23	3.5	4.2
pH of feed: 5.5							
Minimal medium							
Phosphate: 1 m*M*	Phosphate	0.045	4.0	97	24	1.1	4.0
Methanol: 24 g/L		0.10	3.5	25	7.5	0.75	4.0
pH controlled at 4.0							

TABLE 3
Influence of Buffer and pH on Growth and Indoles Production in a Washed-Cell System

Buffer	Initial pH	Minimum pH[a]	Maximum KU[a]	Indoles, mg/L
Without buffer	4.3	2.9	980	76
MES 70 m*M*	5.1	3.3	1180	38
MES 70 m*M*	6.3	6.0	1320	38
HEPPS 70 m*M*	7.5	6.7	1040	234
HEPPS 70 m*M*	9.0	7.6	480	22

[a]We indicate minimum pH and maximum KU obtained because some lysis occurred at the end of the third day. The initial KU was 560.

nine (54%), L-valine (47%), L-aspartic acid (40%), L-leucine (37%), L-glutamic acid (36%), L-glutamine (31%), and L-cysteine (31%).

In the WCS described above, there was an 80–100% increase in DCW in 6 days, presumably owing to growth on endogenous substrates and phosphate carried over from the seed medium. The addition of different antibiotics was investigated and it was found that cycloheximide decreased the growth and increased specific indoles productivity.

The results with cycloheximide are shown in Fig. 5. With 1 g/L cycloheximide, growth was completely prevented and indoles production was the same as the control up to 38 h when it stopped completely. On the other hand, the addition of 50 mg/L cycloheximide slowed growth, but increased indoles production; maximum specific productivity was increased from the control value of 1.2–2.0 mg/g · h.

Discussion

H. polymorpha strain 3-136 has the ability to synthesize a mixture of indole-containing metabolites from methanol. We found this mixture to contain about 10–20% L-tryptophan according to bioassay results using *Lactobacillus arabinosus* ATCC 8014 (Difco Manual, 9th ed., p. 235, 1977). The rest of the indoles are tryptophan metabolites shown recently (*6*) to be mainly tryptophol, indoleacetic acid, and indoleacetaldehyde.

In the present work, we have found indoles production to be markedly affected by environmental conditions such as phosphate concentration, pH, amino acids such as methionine, and a protein synthesis inhibitor such as cycloheximide.

The batch culture experiments revealed that (a) a high methanol concentration (24 g/L) caused a growth lag; (b) indoles production was growth-associated; and (c) that the drop in pH which accompanied growth apparently interfered with indoles production unless the phosphate concentration was on the verge of growth limitation. Indeed, the lowering of phosphate from 66 to 6.6 m*M* doubled volumetric and specific indoles production (Table 1, Fig. 3). Although reduction of phosphate

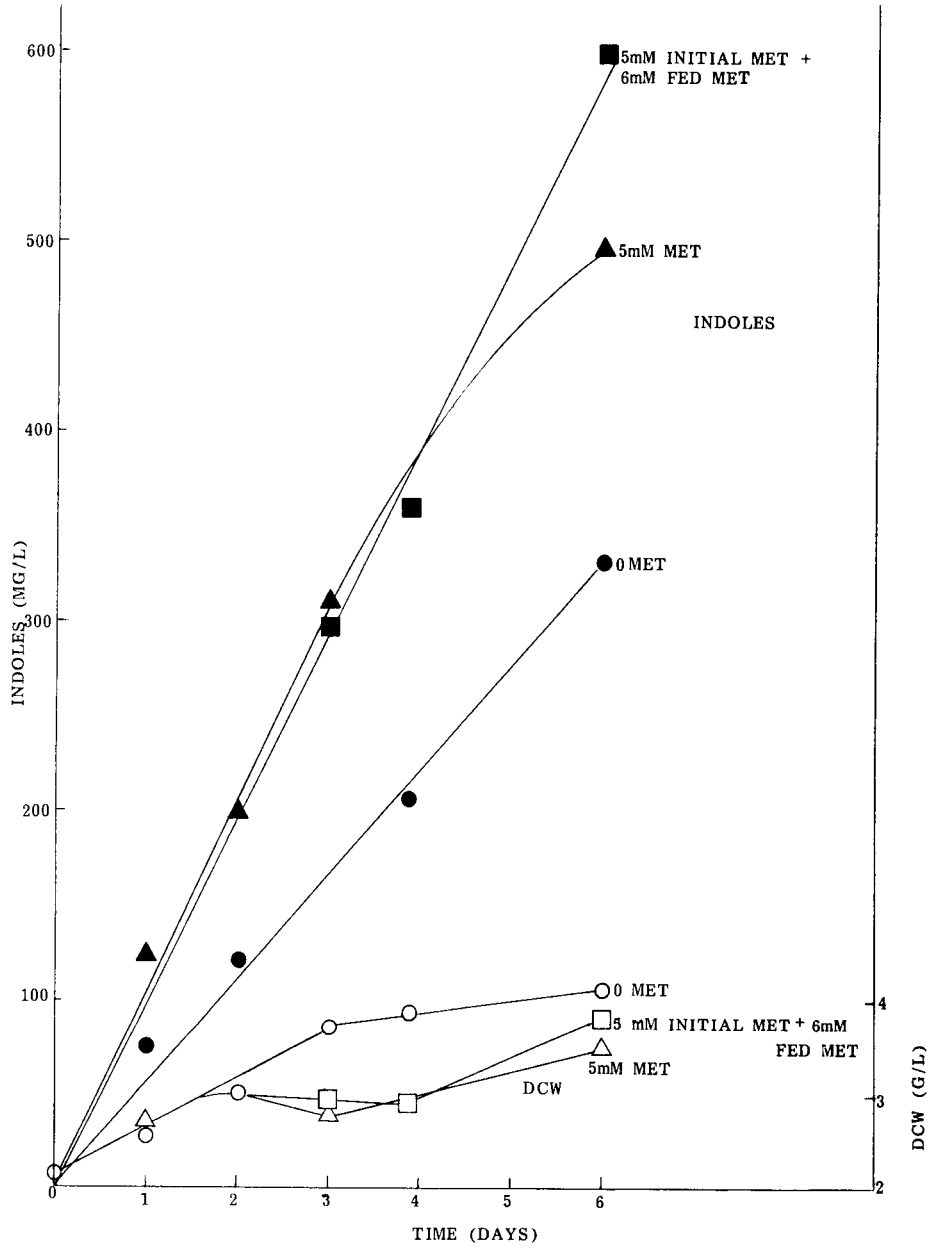

Fig. 4. Growth and indoles production in a 6-day washed-cell system experiment with or without addition of methionine.

to a low level of 0.4 m*M* increased volumetric production by only 30% (as compared to the 66 m*M* phosphate results), the specific production was tripled. Possibly the phosphate effect noted here is related to the observation that intracellular levels of inorganic phosphate regulate both catabolic and biosynthetic enzymes (*1*, *4*).

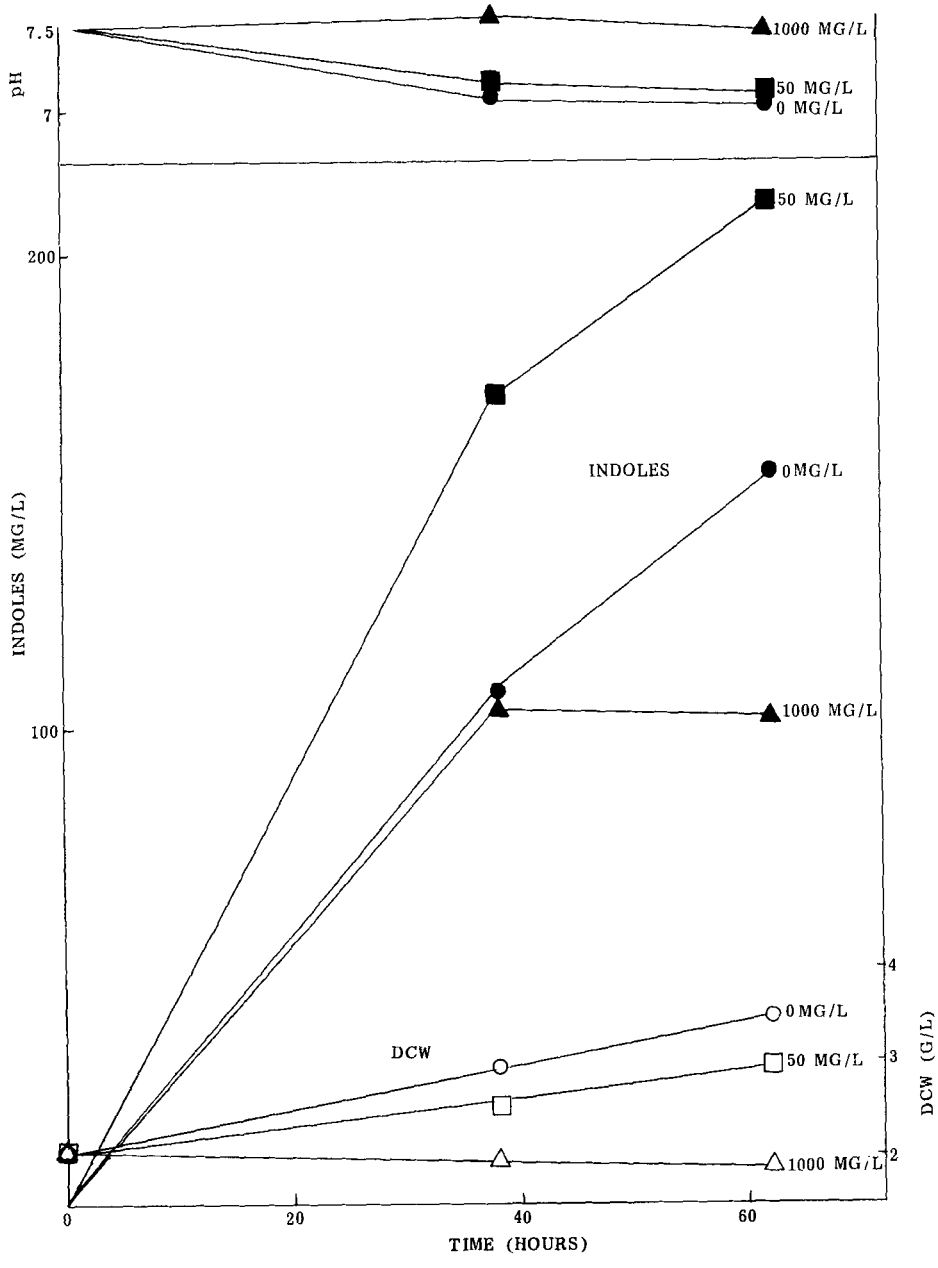

Fig. 5. Effect of added cycloheximide in washed-cell system experiments.

Studies in the chemostat confirmed the importance of phosphate limitation in indoles production. At the same approximate growth rate (0.045–0.05 h^{-1}), the phosphate-limited chemostat produced a volumetric titer of indoles four times that of the carbon-limited chemostat, although the specific titers were the same. The steady-state pH values were similar (4.0 and 4.2) in the two systems. In both types of chemostats, increases in dilution rate lowered indoles production, a surprising result in view of the growth-associated nature of production in batch culture. Inter-

estingly, this negative effect of increased growth rate was prevented when 0.05% yeast extract was added, resulting in the highest specific productivity observed in this study (3.5 mg indoles/g DCW · h).

The lack of correlation between indoles production and growth rate noted in the chemostat stimulated us to attempt production in a WCS. Although some growth did occur in this system because of carryover of phosphate from the seed medium and to endogenous phosphate, it did not amount to even a doubling of biomass. The pH was held fairly constant by 125 m*M* HEPPS buffer at pH 7.45. Both volumetric and specific indoles production were higher by severalfold in this phosphate-limited WCS than in batch culture. The further addition of methionine markedly increased volumetric production to a high of 600 mg/L and specific production to a high of 158 mg/g DCW. The effect was not specific for methionine, although no other amino acid was superior to it; threonine was equivalent to methionine in effect. Perhaps the effect arises from a requirement for amino nitrogen and the active amino acids are the ones best able to contribute their amino group. The stimulation observed by amino acid addition represents about 1 mol indoles/1–2 mol of amino acid; since the indoles contain 1–2 mols N/molecule, the stimulation may be caused by a stoichiometric effect rather than a regulatory effect. It is tempting to speculate that the amino acid effect observed is related to the yeast extract effect noted in the chemostat.

The low level of growth observed in the WCS can be further depressed by addition of 50 μg cycloheximide/mL, resulting in a doubling in indoles specific production. Although complete cessation of growth using 1000 μg cycloheximide/mL interfered with production, it is still possible that an intermediate concentration could completely inhibit growth and yield even better indoles production.

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