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Effect of Matallic Ions, B-Vitamins, & Amino Acids on the Production of Trans-2, 3-Epoxysuccinic Acid by Paecilomyces Varioti

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Eleanor Tsan Mei Ling August, 1976 27 pages

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The nutritional conditions which permit maximum yield of trans-2,3-epoxysuccinic acid by *Paecilomyces varioti* NRRL 1123 were investigated. Copper and iron ions were added to decationized fermentation media to determine the optimum concentration and ratio of the ions for the accumulation of trans-2,3-epoxysuccinic acid. B-vitamin and amino acid studies were done by single omission of the growth factors in culture media. Organic acids in the cultures were recovered by ether extraction and identified by gas chromatography.

In addition to trans-2,3-epoxysuccinic acid, succinic acid, malic acid, and tartaric acid were also found in culture filtrates. The results indicated that with a copper-iron ion ratio and concentration of 1 mM:2 mM, there was a maximum yield of trans-2,3-epoxysuccinic acid while the growth of the fungus was slightly inhibited. Deficiencies in biotin, thiamine, glutamic acid, cystine, aspartic acid, or histidine had marked effects on the production of trans-2,3-epoxysuccinic acid but not on fungal growth.

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INTRODUCTION

Trans-2,3-epoxysuccinic acid (ESA), a dicarboxylic acid having an epoxy ring, was first isolated as a metabolite of *Paecilomyces varioti* and *Penicillium viniferum* (21). Subsequently, its production by *Aspergillus fumigatus* (3), *Byssochlamys nivea* (2) and *Lentinus degener* (17) was established. Following the isolation of the acid as a metabolic product, Payne and Williams (18) synthesized ESA by tungstate-catalyzed hydrogen peroxide oxidation of fumaric acid.

A variety of microorganisms convert ESA into a mixture of meso-tartaric acid and L-(+)-tartaric acid. These compounds are used commercially in bakery, textile, and pharmaceutical industries. Meso-tartaric acid is also synthesized *in vitro* by hydration of ESA at 121 C under pressure (15 lbs per sq-in). The cis and meso isomers of ESA have been prepared by hydrogen peroxide oxidation of benzoquinone and by nitric acid oxidation of carbomycin. The cis isomer can also be prepared by tungstate catalyzed hydrogen peroxide oxidation of maleic acid (13).

When ESA is heated to 42 C in aqueous ammonia, β-hydroxyaspartic acid is formed. β-hydroxyaspartic acid has been shown to be a bacterial mutagen, and, in addition, competitively inhibits aspartate aminopherase. Another derivative of ESA, N,N'-Bis-n-decylepoxysuccinamide, has been used as a specific insecticide for pea aphids. Propargl(-)-trans-epoxysuccinic acid has notable antifungal activity. Erythro-β-hydroxy-L-aspartic acid has been reported to be bactericidal for Gram-negative bacteria grown on Witkin synthetic medium (14). Since ESA is an intermediate in the

production of a variety of compounds which have commercial potentials, improvement of its production and accumulation by a fermentative method would be of great commercial importance.

The addition of refiners blackstrap molasses to the growth media of A. fumigatus has resulted in an increase in yield of ESA over those cultures to which molasses had not been added (15). Blackstrap molasses functions as a source of fermentable sugars, essential minerals (magnesium, zinc, iron, copper, potassium, and phosphorus), B-vitamins (biotin, pantothenic acid, inositol, pyridoxal, and thiamine), and amino acids (principally asparagine, aspartic acid, alanine, glutamic acid, and glycine) (19).

Dibble (5) has demonstrated that the inclusion of iron and copper ions into previously decationized fermentation media containing molasses results in an increased accumulation of ESA. The objective of this research was to further investigate the nutritional conditions which permit maximum yield of ESA, namely, the effect of the balance of ions of copper and iron, B-vitamins, and amino acids on its accumulation.

LITERATURE REVIEW

Trans-2,3-epoxysuccinic acid (ESA) can be produced from glucose, sucrose, fructose, mannitol, sorbitol, xylose, erythritol, glycerol, and ethanol by Paecilomyces varioti (21). When P. varioti was grown on a 10% glucose salts broth, a 10%-15% yield of the acid was recovered by Sakaguchi and Tada (21). Martin and Foster (12) reported a 21% yield of ESA with A. fumigatus on 3% glucose for four days. Moyer (15), by adding 3% methanol to a 14% glucose nutrient-salts broth inoculated with A. fumigatus, increased the yield to 32%. Anzai and Curtis (2) recrystallized approximately 400 mg of acid per liter from culture filtrates of Byssochlamys nivea.

In studies with P. varioti, Normura, Takahashi and Sakaguchi (16) observed that carboxyl groups of ESA produced in the presence of $C^{18}O_2$ had the same specific activity as the average values of carbon atoms 1 and 6 of citric acid produced in the same system. On the basis of these observations, they postulated that oxaloacetic acid was a common precursor for both citric acid and ESA. Martin and Foster (12) postulated that malic acid was the immediate precursor of ESA, just as it was known to be the precursor of oxaloacetic acid. Aida and Foster (1) observed that $^{18}O_2$ was incorporated into ESA and concluded that the epoxide group of this acid is derived directly from molecular oxygen.

Tracer studies with fumarate-1,4-^{14}C and fumarate-2,3-^{14}C indicated that the carbon skeleton of fumarate is incorporated into ESA without change. From these observations, Wilkoff and Martin (26) concluded that

fumarate is the immediate precursor of ESA; however, the oxygenase responsible for this reaction has not been isolated. Cycloheximide and 8-azaguanine have been shown to inhibit the production of the acid, and it was assumed that the oxygenase enzyme was absent or inhibited under these conditions (17).

In glucose-grown cultures of A. fumigatus, meso-tartaric acid was formed enzymatically from ESA (12). This reaction is a hydrolytic reaction and is catalyzed by trans-succinicepoxide hydrolase which apparently is inducible. The induced synthesis of this enzyme takes place at an extremely rapid rate. Meso-tartrate ordinarily does not accumulate in cultures attacking ESA, which indicates that it is utilized as rapidly as it is formed. It is well established that mesotartrate is dehydrated by bacteria to oxaloacetate. In animal mitochondria and Pseudomonas, mesotartrate is dehydrogenated to form oxaloglycolate (7, 26).

Hampel (7) has studied the degradation of tartrate and the various stereoisomers of tartaric acid by Aspergillus niger, Penicillium chrysogenum, Botrytis cinerea and Fusarium semitectum into glyoxlic acid, mesoxalic acid, and oxalglycolic acid in submerged cultures. With mycelial extracts of Fusarium semitectum, a pyridine nucleotide-linked enzymatic oxidation of tartaric acid was demonstrated (7). Oxaloglycolic acid, resulting from the dehydrogenation of tartaric acid, was metabolized by mycelial preparations from Fusarium semitectum in an enzyme-catalyzed reaction with molecular O_2. The formation of ESA and its further oxidation or dehydrogenation by these organisms indicates that it is probably an intermediate in a modified respiratory pathway which may represent a means of bypassing the direct oxidation of malate to oxaloacetate or an alternate version of the glyoxylate cycle.

To improve the production of ESA, one has to either increase the accumulation of the metabolic intermediate or prevent its destruction. This can be accomplished by inhibiting the enzyme system of any side reactions or by inhibiting the enzymes involved in ESA destruction. By regulating the metallic cation content of the fermentation medium, the enzyme systems in which they function might be affected accordingly. Dibble (5) demonstrated that the inclusion of copper and iron ions in decationized media resulted in an increased accumulation of ESA and succinic acid similar to those increases obtained in non-decationized media. These results indicated that the accumulation of ESA is analogous to the fermentative production of citric acid. The addition of ferric ions to synthetic media increased the production of citric acid since iron is required as a cofactor for the enzyme aconitase. The use of copper ion as an antagonist or iron in the fermentation media resulted in an increased yield of citric acid (23).

Research on the structure and function of B-vitamins has established that B-vitamins are integral parts of numerous enzymatic systems. The vitamins are either coenzymes, essential components of coenzymes, or prosthetic groups of enzymes (Table I) (25). The presence or absence of B-vitamins in culture media may affect the growth of fungi (27). Fungi often require an exogenous source of vitamins, in particular, biotin, thiamine, and pyridoxal (10). Addition of nicotinic acid, pantothenic acid, or inositol has not shown any auxithallic property for many fungi (9). In addition to some of the known B-vitamins, amino acids may be important limiting factors in the nutrition of fungi. Amino acids function as the source of nitrogen and several of them are growth-inducing substances and serve as building units for protein synthesis.

TABLE I

SOME COENZYMES AND PROSTHETIC GROUPS OF ENZYMES

Coenzyme or Prosthetic Group	Enzymic and Other Functions	Essential Vitamin
Biotin	CO_2 transfer	Biotin
Coenzyme A	Acetyl or other acyl group transfer; fatty acid synthesis and oxidation	Pantothenic acid
Flavin Adenine Dinucleotide	Hydrogen carrier	Riboflavin
Flavin Mono-nucleotide	Hydrogen carrier	Riboflavin
NAD	Hydrogen carrier	Nicotinic acid
NADP	Hydrogen carrier	Nicotinic acid
Pydidoxal Phosphate	Aminotransferases, amino acid decarboxylase, racemases	Pyridoxal
Thiamine Pyrophosphate	Oxidation decarboxylation, active aldehyde carrier	Thiamine
Inositol	α-amylase	Inositol

MATERIALS AND METHODS

Culture Procedures

Paecilomyces varioti NRRL 1123 was the organism employed throughout this research project. The culture was obtained from Dorothy Fennel of the Northern Regional Research Laboratory, Peoria, Illinois.

Preparation of Standard Spore Inoculum. A loopful (5 mm in diameter) of spores and mycelia of *Paecilomyces varioti* NRRL 1123 previously grown on Sabouraud dextrose agar slants was transferred to each of 20 sterilized screw-top culture tubes (20 x 150 mm) which contained 4.0 gm of corn bran and 6.0 ml of distilled water. The cultures were incubated at 30 C with the screw tops loose for 10 days until abundant spores had developed. The resulting cultures were designated as stock cultures and stored at 5 C for subsequent use.

To prepare a standard spore inoculum, 10 ml from a 100-ml volume of 0.6 mM potassium phosphate buffer (pH 7.2) were added to a corn bran stock culture and allowed to stand for a few minutes. The corn bran was gently pressed with a sterilized pipette to release spores into the buffer solution. Five ml of the supernatant were returned to the initial phosphate buffer. The resulting spore suspension was used as the standard spore inoculum. One ml of the spore suspension was used to inoculate each 50 ml of culture medium (4).

Fermentation Medium. Basal fermentation media for metallic ion studies consisted of the following ingredients made to one liter with

glass-distilled water: 200 gm decationized refiners blackstrap molasses; 0.25 gm $MgSO_4 \cdot 7H_2O$; 2.50 gm $(NH_4)_2SO_4$; 0.30 gm KH_2PO_4; 0.44 gm $ZnSO_4 \cdot 7H_2O$. The media were dispensed in 50 ml quantities into 250-ml Erlenmeyer flasks and sterilized by autoclaving at 121 C for 15 min. To each 50 ml of basal medium, copper and iron ions were added. Sterile $CaCO_3$ (4.0 gm) was added to each flask prior to inoculation.

Decationization was performed by passing a 35% aqueous solution of molasses (v/v) through a glass chromatographic column packed with Amberlite IR 120 resin in the hydrogen form. The column contained 1.0 lb of resin with a volume of 0.02 cu ft of resin. Sugar concentration of the decationized solutions was determined by a modification of the method of Shaffer and Hartman (24). Prior to the sugar assay, the decationized molasses sample was converted to reduced sugar by acidulating with a few drops of 1N H_2SO_4.

Basal fermentation media for the B-vitamin and amino acid studies had the following components made up to one liter: 150 gm decationized glucose monohydrate; 0.25 gm $MgSO_4$; 0.70 gm $(NH_4)_2SO_4$; 0.30 gm KH_2PO_4; 0.44 gm $ZnSO_4 \cdot 7H_2O$. The media were dispensed in 50-ml quantities into 250-ml Erlenmeyer flasks and sterilized in an autoclave at 121 C for 15 min. B-vitamins and amino acids were added to each 50 ml of basal medium by the single omission method. Vitamin solutions were sterilized by filtration using Millipore filters; amino acids solutions were sterilized by autoclaving at 121 C for 15 min. Four grams of sterile $CaCO_3$ were added to each flask prior to inoculation. The glucose monohydrate was decationized as previously described. The concentration of sugar in decationized glucose solution was determined by the modified method of Shaffer and Hartman (24).

Isolation of Fungal Metabolites

Harvest of culture. Prior to harvesting mycelia, a few drops of 1% octadecanol in ethanol (w/v) were added as an anti-foaming agent, and the pH of each culture was adjusted to 1.5 with concentrated H_2SO_4 to free all organic acids. Each culture was filtered through two layers of cheesecloth. The mycelia were washed twice with glass-distilled water, and the washes were added to the culture filtrate. The volume of the culture filtrate was adjusted to 100 ml with distilled water and centrifuged at 16,300 xg for 10 min. The supernatant was extracted with ether. Mycelial weights were determined after drying the mycelia in petri dishes at 100 C for 10 hours.

Extraction of culture filtrates. After centrifugation, the supernatant from the culture filtrates was extracted with ethyl ether in a continuous extractor. For the metallic ion studies, two-hour extraction periods were employed. A four-hour extraction period was used in the vitamin and amino acid nutritional studies. The extracts were evaporated to dryness at room temperature and recrystallized from ether-chloroform (2:1 v/v) (17). The samples were placed in a drying oven at 100 C for 10 min to evaporate any residual water.

Methylation and Gas Chromatography

Samples were methylated with freshly prepared diazomethane generated from Diazald (Aldrich Chemical Corporation, Inc., Milwaukee, Wisconsin). The samples were then allowed to evaporate to dryness at ambient temperature. Prior to gas chromatography, 5.0 ml of ether were added to the samples of which 25 µl were injected into the gas chromatograph.

The gas chromatograph used was a Varian Aerograph Model 200 equipped with a 5' x 0.25" copper column. The column contained 10% polyphenyl ether

as the stationary phase with Anakron ABS (80/90 mesh) as the support. The following chromatographic conditions were employed throughout this study: (1) a gas flow rate (helium) of 120 ml/min; (2) an injector temperature of 210 C; (3) a detector temperature of 225 C; (4) a column temperature of 170 C. The chromatograph was equilibrated prior to use by heating at 170 C for 24 hours under a continuous flow of helium. Samples (25 µl) were applied by on-column injection. The total time for chromatographic separation of each sample was 24 min.

Standardization of Known Organic Acids

Known quantities of malic acid, succinic acid, oxalacetic acid, tartaric acid, fumaric acid and ESA were methylated as previously described. Their retention times were determined by gas chromatography. Mean peak areas of samples run in triplicate were also determined for each methyl ester. The ESA was a gift of the Northern Regional Research Laboratory, Peoria, Ill. Fumaric acid was obtained from J. T. Baker Chemical Company, Phillipsburg, New Jersey; meso-tartaric acid from Nutritional Biochemical Corp., Cleveland, Ohio; succinic acid from Mallinckrodt Chemical Works, St. Louis, Missouri; malic acid from J. T. Baker Chemical Co., Phillipsburg, New Jersey; and oxalacetic acid from Sigma Chemical Company, St. Louis, Missouri.

Gas Chromatography Using Trimethylsilyl Derivatives

The gas chromatographic method employed in this investigation for the determination of ESA requires the esterification of the organic acids with diazomethane. Although the use of diazomethane is simple, it is toxic, explosive and has a relatively short storage life. The need for a more efficient esterification procedure for quantitation of ESA was apparent;

therefore, the use of trimethylsilyl (TMS) derivatives for the chromatographic assay of ESA was investigated.

A simple and rapid quantitative method for the preparation of trimethylsilyl (TMS) derivatives of organic acids for gas chromatography has been described by David, Heinz, and Addicott (4). TMS derivatives of ESA and other organic acids recovered from culture filtrate were prepared. The TMS esters were injected into the gas chromatograph containing 5' x 0.25" copper column packed with either 30% SE-30 or 15% SE-52. Unsatisfactory resolution was obtained after repeated attempts under the described conditions, and the method was abandoned.

RESULTS AND DISCUSSION

Effect of Copper and Iron

A series of 250-ml Erlenmeyer flasks which contained 50 ml of decationized molasses-salt medium was prepared. Copper and iron ions were added to the medium as $CuSO_4$ and $FeCl_3$, respectively. The Cu^{++}/Fe^{+++} ion concentrations employed are shown in Table 2. Cultures at each concentration of copper and iron ions were made in triplicate. The culture media were inoculated with a standard spore inoculum of *P. varioti* and incubated on a rotary shaker at 30 C for 7 days. The metabolites were extracted from the cultures and methylated according to the procedures mentioned previously. Gas chromatographic identification and quantitation of each metabolite in the samples were determined by comparison to the known standards.

The results showed that significant amounts of ESA and succinic acid accumulated in many of the cultures (Table 2). Cultures containing a Cu^{++}/Fe^{+++} ratio of 1 mM:2 mM resulted in the greatest yield of ESA while 0.001 mM:1 mM of Cu^{++}/Fe^{+++} resulted in the highest yield of succinic acid. There was a decrease in growth of the fungus (as indicated by mycelial weight) as the concentration of copper increased which resembles the response of *A. niger* in citric acid production (23). Fungal growth was totally inhibited at iron concentrations less than 1 mM or at copper concentrations greater than 2 mM. A molar ratio of Cu^{++}/Fe^{+++} of 1 mM:2 mM appeared to be the optimum ratio for production of ESA under the experimental conditions employed. The other molar concentrations and ratios

TABLE 2

EFFECT OF CU AND FE IONS ON THE GROWTH OF *PAECILOMYCES VARIOTI* AND THE ACCUMULATION OF ESA AND SUCCINIC ACID

Cu:Fe (mM)	Mycelial Weight (Gram/Culture)	ESA (Gram/Culture)	Succinic Acid (Gram/Culture)
0.001 : 1	13.06	0.0020	0.0420
0.010 : 1	13.28	0.0358	0.1664
0.100 : 1	11.57	0.0380	0.0600
1 : 1	9.12	0.0480	0.0664
1 : 0.001	0.00	0.0000	0.0000
1 : 0.010	0.00	0.0000	0.0000
1 : 0.100	0.00	0.0000	0.0000
1 : 2	8.47	0.1140	0.0180
1 : 5	7.39	0.0000	trace*
1 : 10	1.60	0.0000	0.0000
2 : 1	0.00	0.0000	0.0000
5 : 1	0.00	0.0000	0.0000
10 : 1	0.00	0.0000	0.0000

* less than 2 mg

of Cu^{++} and Fe^{+++} may have enhanced subsequent metabolism of ESA or resulted in a decrease in its formation. With a proper balance of copper and iron ions in the fermentation medium, fungal growth may have been slightly inhibited resulting in an increase in the accumulation of ESA (Fig. 1).

To further define the Cu^{++}/Fe^{+++} concentration, additional experiments were designed. In these experiments, the concentration of Cu^{++} in all cultures was 1 mM while the concentration of Fe^{+++} varied from 1 mM to 3 mM with increments of 0.5 mM. The data obtained showed that a difference in the iron concentration of 0.5 mM did not affect the fungal growth significantly but had pronounced effect on the accumulation of ESA and succinic acid (Table 3). Again, there was a maximum accumulation of ESA with a Cu^{++}/Fe^{+++} of 1 mM:2 mM.

The next experiment was performed to determine the proper molar concentration of the ions with a Cu^{++}/Fe^{+++} ratio of 1:2. A series of cultures was prepared to contain concentrations of copper and iron ions, respectively, of 0.5 mM:1 mM, 1 mM:2 mM, 2 mM:4 mM, 3 mM:6 mM, 4 mM:8 mM, 5 mM:10 mM. Molar concentrations of 0.5 mM:1 mM, 1 mM:2 mM, and 2 mM:4 mM had little effect on mycelium production, but the maximum accumulation of ESA occurred with a Cu^{++}/Fe^{+++} molar concentration of 1 mM:2 mM (Table 4). There was a significant decrease in mycelial weight and acid production when the copper concentration exceeded 2 mM. No acids were accumulated when the Cu^{++} concentration was above 3 mM. With a concentration of 5 mM: 10 mM/copper:iron, growth of fungus was totally inhibited.

The results of the preceding experiments indicated that with a ratio and concentration of copper and iron ions of 1 mM:2 mM results in a maximum accumulation of ESA by P. varioti while mycelial growth is slightly inhibited. The data also indicated that as ESA accumulation increases,

Figure 1. Effect of Cu and Fe ions on the growth of Paecilomyces varioti and the accumulation of ESA.

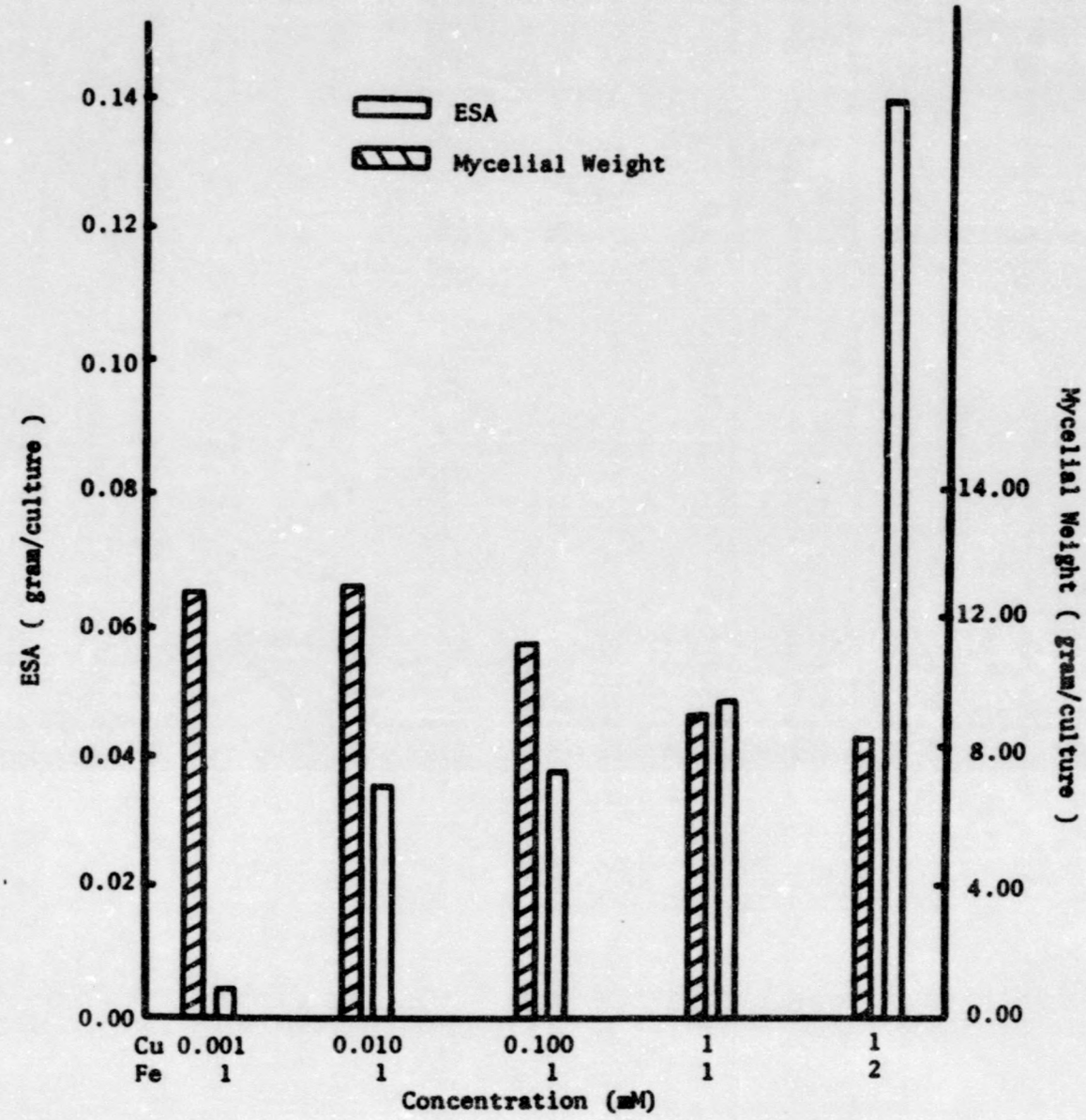

ESA
Mycelial Weight
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
ESA (gram/culture)
14.00
12.00
8.00
4.00
0.00
Mycelial Weight (gram/culture)
Cu 0.001 0.010 0.100 1 1
Fe 1 1 1 1 2
Concentration (mM)

TABLE 3

EFFECT OF CU AND FE IONS ON THE GROWTH OF *PAECILOMYCES VARIOTI* AND THE ACCUMULATION OF ESA AND SUCCINIC ACID

Cu:Fe (mM)	Mycelial Weight (Gram/Culture)	ESA (Gram/Culture)	Succinic Acid (Gram/Culture)
1 : 1	18.11	0.0221	0.0043
1 : 1.5	18.16	0.0180	0.0020
1 : 2.0	19.61	0.0514	trace
1 : 2.5	21.14	0.0167	trace
1 : 3.0	20.38	trace	0.0000

TABLE 4

EFFECT OF CU AND FE IONS ON THE GROWTH OF PAECILOMYCES VARIOTI AND THE ACCUMULATION OF ESA AND SUCCINIC ACID

Cu:Fe (mM)	Mycelial Weight (Gram/Culture)	ESA (Gram/Culture)	Succinic Acid (Gram/Culture)
0.5 : 1	19.43	0.0151	0.0025
1 : 2	20.36	0.0704	0.0012
2 : 4	18.72	0.0085	0.0016
3 : 6	3.14	trace	trace
4 : 8	1.80	0.0000	0.0000
5 : 10	0.00	0.0000	0.0000

succinic acid accumulation is relatively low. Hence, it appears that a proper concentration of copper and iron ions will enhance the metabolism of succinic acid to ESA, or inhibit the further destruction of tartrates resulting in an increase in ESA accumulation. An improper ratio and concentration of the two ions enhanced the accumulation of succinic acid or the further metabolic destruction of tartrate and thus a decrease in ESA accumulation.

Mahansmith et al. (11) have shown that the inclusion of 1.0 mM of ferric ions in fermentation media prevented tartrate utilization by Aspergillus flavus. Hulbert and Jakoby (8) demonstrated that tartrate dehydrase from Pseudomonas putida required ferrous ions for activity. It is not possible at this time to indicate which specific enzyme-catalyzed reactions are being affected by the copper and iron ions since the pathways of ESA metabolism in Paecilomyces varioti have not been identified or the enzymes isolated. Therefore, further investigations are necessary to develop a unified theory of the possible mechanisms for the metabolism of ESA.

Effect of B-vitamins

These experiments were designed to determine which B-vitamin, or combinations of several B-vitamins, were essential for the maximum production of ESA by P. varioti. Eight vitamins were used in this study. A series of 250-ml Erlenmeyer flasks which contained 50 ml of decationized glucose monohydrate-salts medium was prepared. To this basal medium 1 mM of Cu^{++} and 2 mM of Fe^{+++} were added. By the single omission method, each experimental culture was deficient in one of the eight B-vitamins. The quantity of each of the B-vitamins in 50 ml of culture medium was:

aminobenzoic acid	50 mμ moles
pantothenic acid	50 mμ moles
inositol · $2H_2O$	50,000 mμ moles
niacinamide	50 mμ moles
pyridoxal HCl	50 mμ moles
riboflavin	50 mμ moles
thiamine HCl	50 mμ moles
biotin	0.41 mμ moles

Two controls were used, one deficient in all B-vitamins and the other to which all had been added. All controls and experimentals were made in triplicate. The flasks were inoculated with a standard spore inoculum. The cultures were incubated on a rotary shaker at 30 C for 7 days. Mycelial weights and organic acids were determined by the methods described earlier.

The data show that the omission of all B-vitamins resulted in a complete cessation of growth (Table 5). There was an optimal yield of ESA when all eight B-vitamins were supplied. The absence of either thiamine or biotin from the media gave a significantly lower yield of ESA. Hence, these two vitamins are apparently the most essential for the maximum accumulation of ESA under the growth conditions employed. Riboflavin, inositol, pyridoxal, nicotinic acid, and p-aminobenzoic had an intermediate effect on ESA accumulation while pantothenic acid had the least effect (Fig. 2).

In addition to ESA and succinic acid, malic acid was also found in a few of the cultures. When either p-aminobenzoic, riboflavin, thiamine, pyridoxal, or biotin was not supplied, malic acid was not detected. Since the omission of any one of the vitamins did not inhibit growth of the fungus, the vitamins must have affected the metabolism of the matured mycelium. Konishita *et al.* (19) postulated that biotin, in addition to limiting growth, may also operate by altering metabolic pathways. For example, with a biotin deficiency succinic acid oxidation was found to

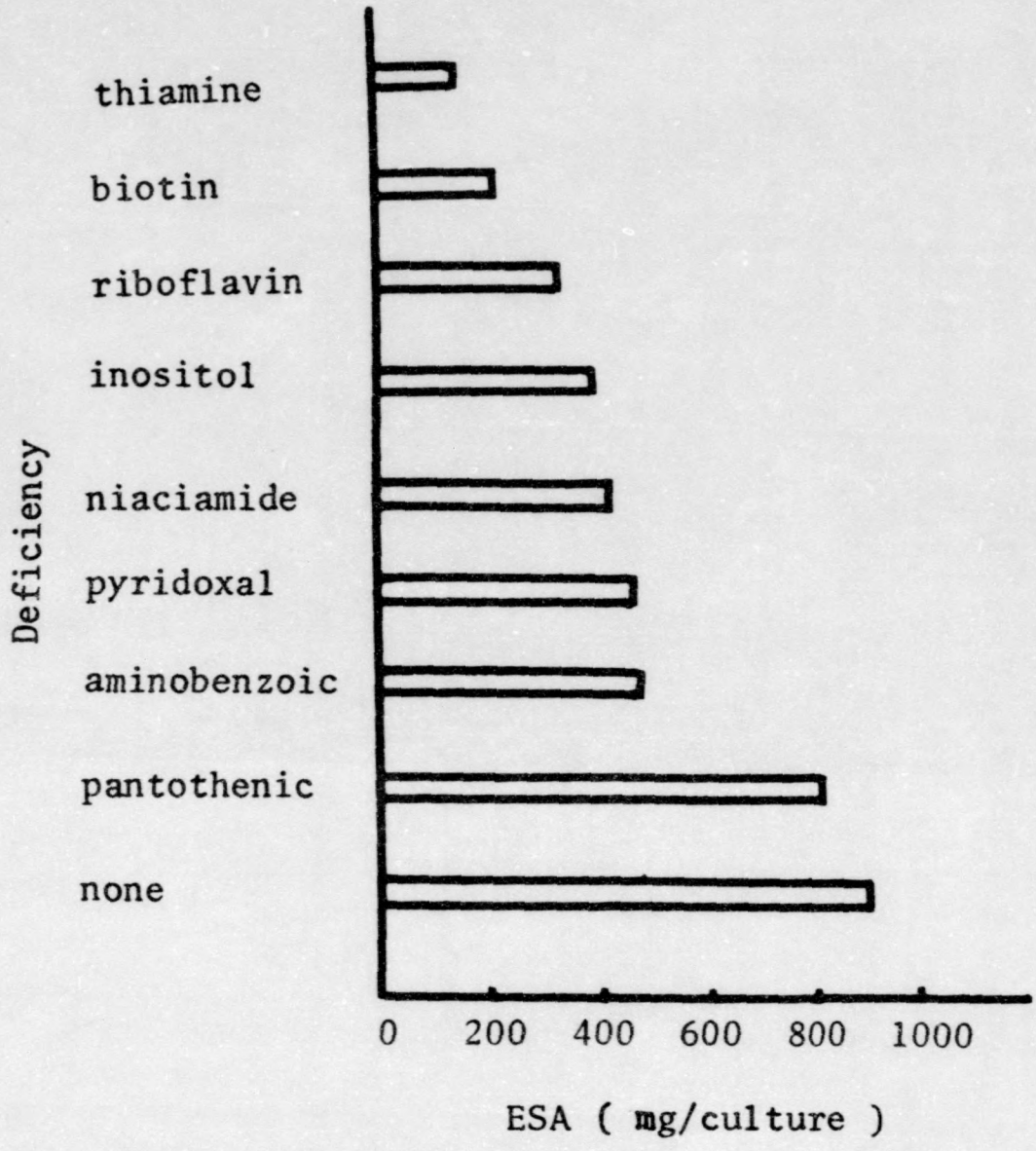
TABLE 5

EFFECT OF B-VITAMINS ON THE PRODUCTION OF ESA, SUCCINIC ACID, MALIC ACID AND MYCELIAL WEIGHT BY PAECILOMYCES VARIOTI

Media	Mycelial Weight	ESA (Gram/Culture)	Succinic Acid	Malic Acid
No vitamins	0.00	0.0000	0.0000	0.0000
All vitamins	19.20	0.9161	0.0583	0.0514
- thiamine	17.85	0.1286	0.0299	0.0000
- biotin	20.28	0.1929	0.0199	trace*
- riboflavin	19.54	0.3439	0.0204	0.0000
- inositol	18.55	0.3703	0.0351	0.0176
- nicotinic acid	18.53	0.4114	0.0207	0.0282
- pyridoxal	17.12	0.4499	0.0364	0.0000
- aminobenzoic	18.90	0.4596	0.0329	0.0000
- pantothenic acid	20.18	0.8513	0.0490	0.0132

* trace - less than 2 mg

Figure 2. Effect of B-vitamins on the production of ESA by Paecilomyces varioti.


thiamine
biotin
riboflavin
inositol
niaciamide
pyridoxal
aminobenzoic
pantothenic
none
Deficiency
0
200
400
600
800
1000
ESA (mg/culture)

be decreased. Thus, the flow of carbon to fumaric acid and aspartic acid could be partially blocked. Part of the carbon usually drained off in this pathway can be used for glutamic acid production. The effect of the omission of biotin on the accumulation of ESA may also be a result of this blockage. It is known that biotin, pantothenic acid, riboflavin, nicotinic acid, pyridoxine, and thiamine are coenzymes or prosthetic groups of some enzymes; therefore, the absence of any one of them may impair the activity of certain enzymes (25). As a result, some metabolic pathways of this fungus could have been partially inhibited and this resulted in a decrease in production of ESA. Yeasts have been reported to synthesize riboflavin and p-aminobenzoic acid (6). This may account for the lesser effect of the omission of this vitamin on ESA metabolism of P. varioti. Leonian and Lilly (9) reported that nicotinic acid and pantothenic acid did not show any auxithallic properties for the 25 fungi used in their study. Thiamine is an essential component of thiamine pyrophosphate which is a coenzyme involved in oxidative decarboxylation of α-keto acids. Its deficiency in culture media may affect the metabolism of the fungus and the accumulation of ESA. The exact functions of the B-vitamins on the production of ESA by P. varioti in culture media cannot be explained at this time. Future investigations are necessary to reveal their roles in ESA production.

Effect of Amino Acids

Many studies have related microbial growth and metabolism to the amino acids in the medium. For example, leucine, arginine, histidine, and methionine were found to be growth factors required by many fungi (10). If certain amino acids can be demonstrated to affect the accumulation of ESA, their proper use in culture media may lead to an increase in the yield of ESA.

A series of 250-ml Erlenmeyer flasks containing 50 ml of decationized glucose monohydrate-salts medium was prepared. To the basal medium were added 1 mM of copper and 2 mM of iron ions. To one set of controls, none of the amino acids was added. To another set of controls, all twenty amino acids were added to the basal medium. The concentration of each amino acid was 0.006 mM. By the single omission method, single amino acids were omitted from the experimental cultures. All controls and experimental cultures were made in triplicate. The media were inoculated with standard spore inocula, and the cultures were incubated on a rotary shaker at 30 C for 7 days. Mycelial weights and organic acids in the cultures were determined as described previously.

ESA, tartaric acid, succinic acid, and malic acid were found in the cultures (Table 6). Omission of any one amino acid did not affect fungal growth as indicated by total mycelial weights. With the exception of malic acid, all the organic acids were found in a maximum amount when all the amino acids were present in the culture media. Omission of cystine and glutamic acid from the culture media resulted in no accumulation of ESA, succinic acid, or malic acid, but a trace amount of tartaric acid was detected. Absence of aspartic acid and histidine resulted in slight accumulation of ESA but not succinic acid or malic acid. Omission of asparagine, lysine, isoleucine, or glycine resulted in a similar decrease in ESA production. When arginine, methionine, proline, and valine were absent from the medium, accumulation of ESA was reduced to 16%-27% of the control. Tyrosine, tryptophane, leucine, norleucine, and phenylalanine had intermediate effects while threonine and serine had relatively little effect on ESA accumulation.

TABLE 6

EFFECT OF OMISSION OF AMINO ACIDS ON THE PRODUCTION OF ESA, TARTARIC ACID, SUCCINIC ACID, MALIC ACID, AND MYCELIAL WEIGHT

Media	Mycelial Weight	ESA	Tartaric Acid	Succinic Acid	Malic Acid
No amino acids	0	0	0	0	0
All amino acids	12.91	0.1438	0.0288	0.0410	0.0000
- cystine	11.87	0.0000	trace*	0.0000	0.0000
- glutamic	11.20	0.0000	trace	0.0000	0.0000
- aspartic	11.30	0.0034	0.0000	0.0000	0.0000
- histidine	12.56	0.0042	trace	trace	0.0000
- lysine	10.85	0.0071	0.0000	0.0015	0.0000
- asparagine	11.07	0.0073	0.0000	trace	0.0000
- glycine	10.61	0.0086	0.0057	0.0000	0.0000
- isoleucine	10.71	0.0119	0.0000	0.0019	0.0023
- alanine	12.11	0.0168	0.0120	0.0013	0.0000
- arginine	12.61	0.0236	0.0000	0.0014	0.0000
- methionine	11.23	0.0346	0.0150	0.0029	0.0013
- valine	11.61	0.0359	0.0085	0.0191	0.0038
- proline	11.98	0.0399	0.0125	0.0064	0.0024
- tyrosine	12.02	0.0729	0.0112	0.0169	0.0000
- tryptophane	12.06	0.0771	0.0148	0.0212	0.0015
- leucine	12.01	0.0785	trace	0.0011	0.0016
- norleucine	11.73	0.0837	0.0177	0.0219	0.0025
- phenylalanine	12.28	0.0869	0.0157	0.0195	0.0018
- threonine	11.91	0.1088	0.0209	0.0216	0.0079
- serine	11.75	0.1294	0.0263	0.0304	0.0043

*Less than 2.0 mg.

Alanine can enter the glycolytic pathway via pyruvic acid; aspartic acid and glutamic acid enter the Krebs' cycle via oxaloacetic acid and α-ketoglutaric acid, respectively. By transamination, many of the amino acids can be interconverted. Aspartic acid and glutamic acid also play an important role in purine and pyrimidine synthesis. The exact mechanism of the effect of amino acids on ESA production would be difficult to define at this time. Further investigation of the proper concentrations or a combination of the amino acids in a fermentation medium may result in an optimal yield of ESA.

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