Tetracycline Production with Sweet Potato Residue by Solid State Fermentation

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For saving energy in antibiotic production and reducing the amount of agricultural wastes, solid state fermentation was used in this study to produce tetracycline with sweet potato residue by *Streptomyces viridifaciens* ATCC 11989. It was found that the optimal media for tetracycline production were sweet potato residue 100 g, organic nitrogen (rice bran, wheat bran, or peanut meal) 20 g, $(NH_4)_2SO_4$ 2.4 g, KH_2PO_4 0.4 g, $CaCO_3$ 1.8 g, NaCl 0.6 g, $MgCl_2$ 0.8 g, soluble starch 10 g, methionine 0.2 g, histidine 0.8 g, and monosodium glutamate 1.6 g with initial moisture content 68–72%, and initial pH 5.8–6.0. Each gram of dry weight substrate was inoculated with 1.0×10^8 conidia and incubated at 26°C for 5–7 days, producing 4720 μg of total tetracycline equivalent potency. When incubated at 26°C with the initial moisture content 68%, the conidia in solid media germinated on the second day, mycelia grew abundantly on the third day and reached stationary phase on the sixth day. The antibiotic production was consistent with the morphogenesis of *S. viridifaciens:* activity could be detected on the third day, had the maximal potency on the sixth day, and decreased slightly on the tenth day. (11-3-88 tly)

INTRODUCTION

In 1982, although more than 5500 kinds of antibiotics were reported, most of them had no economic value due to toxicity or ineffectiveness in clinical use.[1] In Taiwan, tetracyclines, cephalosporins, penicillins, and aminoglycosides are the major antibiotics.[2] Most of the raw materials of antibiotics were imported from other countries and their derivatives were prepared in Taiwan.[2]

Tetracyclines are broad-spectrum antibiotics, and the hydrocarbon derivatives of octahydronaphthacene with four annelated 6-membered rings. Submerged culture was usually used for tetracycline production, resulting in much energy input and waste water production.[3,4] In addition, culture media and culture conditions would affect the kind and the quantity of antibiotic production.[4–8]

Sweet potatoes and its residue are abundantly available in Taiwan. In 1985 the cultivation area of sweet potato was 23,239 ha and annual production was 369,461 tons.[9] Starchy materials have a high productivity per hectare and an excellent rate of fermentation by a great number of fast-growing microorganisms.[10–12] With the goal of being economically competitive, sweet potato residue, a starchy agricultural waste, was used in this article to produce tetracyclines with *Streptomyces viridifaciens* by solid state fermentation.

MATERIALS AND METHODS

Sweet Potato Residue

Sweet potato residue was purchased from the local market in Taiwan, screened with 4-16 mesh to remove the dust and large aggregates. It contains 14.0–16.1% moisture, 2.32% crude protein, 3.6% ash, 18.1% crude fiber, and 65.4% carbohydrate.

Tested Organisms

Streptomyces viridifaciens ATCC 11989 was used for tetracycline and chlortetracycline production, and *Bacillus subtilis* ATCC 6633 was used for bioassay of antimicrobial activity.

Culture Media and Culture Conditions

The tested organism was cultivated in a slant of inorganic salt starch agar at 26°C. The medium contained soluble starch 10 g, $(NH_4)_2SO_4$ 2.0 g, $CaCO_3$ 2.0 g, NaCl 1.0 g, K_2HPO_4 1.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, trace-element solution 1 mL (contained $FeSO_4 \cdot 7H_2O$ 1.0 g, $CuSO_4 \cdot 5H_2O$ 0.5 g, $ZnSO_4 \cdot 7H_2O$ 1.0 g and $MnSO_4 \cdot 2H_2O$ 0.1 g/L) and agar 20 g/L.

The basal solid medium contained sweet potato residue 100 g, nitrogen source [$(NH_4)_2SO_4$ 2.4 g, rice bran or wheat bran or soybean meal 20 g], $CaCO_3$ 1.0 g and NaCl 0.2 g. The medium was mixed thoroughly with conidia and distilled water, and incubated statically in flask (the thickness of medium was about 2 cm) at 26°C for 5–7 days by stirring once a day.

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Extraction of Antibiotics

After fermentation, the antibiotic was extracted with 5-times volume of distilled water with shaking at room temperature for five minutes.

Qualitative and Quantitative Determination of Antibiotics

Bioassay

Antimicrobial activity of culture extracts was measured by the paper disc method (diameter 8 mm, Toyo Seisakusho Co., LTD.) with *B. subtilis* ATCC 6633 as the tested organism in antibiotic medium I (Difco Laboratory, USA) at 30°C.

Total tetracycline equivalent potency was calculated from the clear zone of a standard curve of tetracycline (Sigma Co., USA) in the range of 1 μg/mL to 1 $\times$ 10^4 μg/mL.

High Performance Liquid Chromatography Method

Culture extract was filtered with 0.46 μm Millipore filter, and antimicrobial activity was determined by Shimadzu LC-5A Liquid Chromatograph. The operation conditions were with column ODS 120 T and mixture of methanol: acetonitrile: water: pH 2.5 0.2*M* phosphate buffer at ratios of 10:20:60:10 as the mobile phase. The flow rate was 1 mL/min, and the antibiotic was detected with UV detector at wavelength 254 nm or 355 nm. Authenic tetracycline, chlortetracycline, and oxytetracycline were used as the standard for qualitative and quantitative determination in the range of 1 μg/mL to 100 μg/mL.

Observation Under Scanning Electron Microscope

Morphogenesis of tested organism on the solid substrate with different moisture contents or different incubation periods were observed under Hitachi S-550 Scanning Electron Microscope (Hitachi Co., Japan) at 20 KV with gold metal shadowing.[13]

Moisture Content

Samples were dried at 60°C under vacuum for 8–12 hours until their weight remained constant. The weight difference after drying was considered the moisture content.

pH

Initial pH of substrate was determined directly with pH meter, while final pH was determined with 5-times volume of distilled water.

Water Activity

Samples with different moisture contents were placed in a sealed container at 25°C, and water activity was determined by a Hygrometer, or modified Conway method.[14]

Bulk Density

The dry weight or wet weight of samples per unit volume (1 mL) was the bulk density in dry weight or wet weight, respectively.[15]

RESULTS

Assay of Tetracyclines

There are linear relationships between the inhibition zone of *B. subtilis* ATCC 6633 grown in antibiotic medium I at 30°C and the concentration of tetracycline, chlortetracycline, or oxytetracycline in the range of 1 μg/mL to 1 $\times$ 10^4 μg/mL. The antimicrobial activities of chlortetracycline, tetracycline, and oxytetracycline were in the ratios of 2.5:1:0.35 at the same concentration. In addition, there are also linear correlations between the absorbances at wavelength 254 nm or 355 nm in high performance liquid chromatograph (HPLC) and the concentration of tetracycline, chlortetracycline, or oxytetracycline in the range of 1 μg/mL to 100 μg/mL. After 5–7 days fermentation, antibiotic was extracted from the solid medium with 5-times volume of distilled water. Paper disc method was used to calculate the total tetracycline equivalent potency, and HPLC method was used to confirm and calculate the concentration of antibiotic.

Effect of Initial Moisture Content on Cell Morphogenesis

Cell morphogenesis of *S. viridifaciens* grown on the sweet potato residue solid substrate with initial moisture content 68% at 26°C is observed under scanning electron microscope. At zero time, surface of substrate was covered by the conidia. At 1-day incubation, conidia were emerging from germination and tetracycline potency was undetectable. At 2-days incubation, mycelia grew on the surface of substrate in the log phase and tetracycline potency was low, with 24 μg of tetracycline and 45 μg of chlortetracycline per gram dry substrate. At 5-days fermentation, mycelia were in the stationary phase and tetracycline potency was high, with 105 μg of tetracycline and 285 μg of chlortetracycline per gram dry substrate. In 6–7 days incubation, both mycelia and spores were present and tetracycline potency was intermediate, with 81–63 μg of tetracycline and 150–135 μg of chlortetracycline per gram dry substrate.

Cell morphogenesis was also affected by initial moisture content. At low moisture content 58%, both mycelia and

conidia were present on the surface of substrate and produced 220 μg of total tetracycline equivalent potency per gram dry substrate at the end of five days incubation. At 63%, mycelia were the major component on the surface of substrate and produced 350 μg of total tetracycline equivalent potency. At 68%, only mycelia were present and produced 896 μg of total tetracycline potency. At high moisture content 75–80%, both mycelia density and total tetracycline equivalent potency decreased simultaneously.

The correlation between total tetracycline equivalent potency and concentration of tetracyclines is shown in Table I. Chlortetracycline (15.7–31.8%) and tetracycline (10.8–13.5%) as the major antibiotics of *S. viridifaciens*. The ratio of chlortetracycline and tetracycline was affected by initial moisture content. Chlortetracycline content increased with the moisture content. It had the maximal value, 31.8%, at initial moisture content 68%, and decreased to 15.8% at the initial moisture content 80%. Tetracycline content in the total tetracycline equivalent potency was less affected than that of chlortetracycline. Therefore, initial moisture content of substrate was very important in the tetracycline production in solid state fermentation.

Optimization of Tetracycline Production

Sweet potato residue, distilled water, and conidia of *S. viridifaciens* were mixed thoroughly, and cultivated at 26°C for five days. The tetracycline potency was very low and each gram of dry substrate produced 4.5 μg total tetracycline equivalent potency. In order to improve the tetracycline production, the following parameters were investigated.

Mechanism of Tetracycline Secretion

Time course of tetracycline potency and substrate pH with 20% soybean meal as nitrogen source is shown in Figure 1. During fermentation, tetracycline potency was detected at the third day, reached the maximal potency, with 950 μg/g dry substrate, at the fifth day, and decreased gradually from the sixth day to the 30th day. At the 15th day, tetracycline potency was 600 μg/g dry substrate, about 63% of the original antibiotic activity, while at the 30th day, tetracycline potency was less than 15 μg/g dry substrate. Substrate pH decreased sharply from 5.8 to 3.8 in 5-days incubation, then increased slightly from pH 3.8 to 6.9 in 1-month incubation. However, in the case of substrate with 40% soybean meal as nitrogen source, tetracycline potency reached the maximal value, 828 μg/g dry substrate at the sixth day. Tetracycline potency was only 13 μg at the 15th day, and it was not detected after 20-days incubation. The change of substrate pH was similar to that with 20% soybean meal as nitrogen source. Since tetracycline in *S. viridifaciens* was a secondary metabolite, it was secreted at the end of log phase or at stationary phase. Substrate with 40% of soybean meal as nitrogen source stimulated the growth of *Streptomyces* and repressed the biosynthesis of tetracycline. Therefore, 20% of soybean meal as nitrogen source was good enough for cell growth and tetracycline production in this study.

Initial Moisture Content

The effect of initial moisture content of substrate on tetracycline production is shown in Table I. Tetracycline pro-

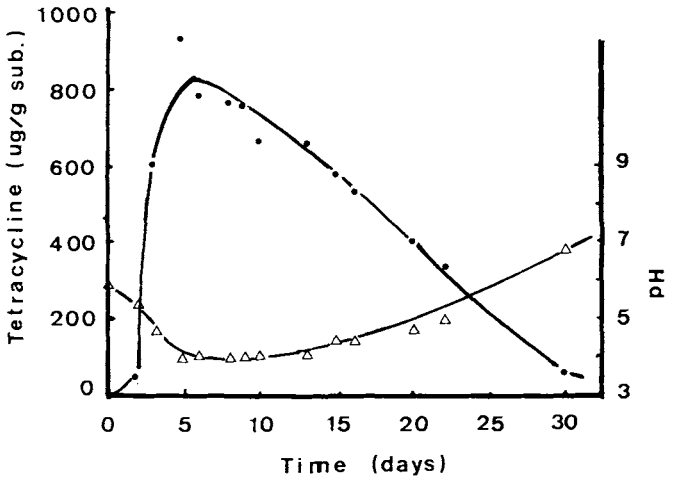

Figure 1. Time course of tetracycline production in solid state fermentation. The solid substrate was adjusted to initial moisture content 68%, and incubated at 26°C. Other conditions were same as Table I. ●—● Total tetracycline equivalent potency, △—△ pH.

Table I. Tetracycline determination with bioassay method and HPLC method at different initial moisture content.[a]

Moisture content (%)	Initial pH	Final pH	Bioassay[b] method (μg/g sub.)	HPLC method (μg/g sub.)	
				TC	CTC
55	6.00	5.90	50	6 (12.0%)[c]	10 (20.0%)
58	6.00	5.90	220	26 (12.0%)	42 (19.0%)
63	6.00	4.60	350	42 (12.0%)	90 (25.7%)
68	6.00	4.00	896	105 (11.7%)	285 (31.8%)
75	6.00	4.10	600	81 (13.5%)	180 (30.0%)
80	6.00	4.20	525	57 (10.8%)	83 (15.8%)

[a] Sweet potato residue 100 g, $(NH_4)_2SO_4$ 2.4 g, soybean meal 20 g, $CaCO_3$ 1 g, NaCl 0.2 g, and 5×10^9 conidia were mixed thoroughly, and incubated at 26°C for five days.

[b] Calculated as total tetracycline equivalent potency with tetracycline content.

[c] Percentage of total tetracycline equivalent potency.

duction increased with moisture content from 55% to 68%. When the initial moisture content was less than 58%, tetracycline production was low as the substrate was too dry for cell growth and antibiotic production. At initial moisture content 68%, Aw of the substrate was 0.995, and bulk densities on dry and wet weight bases were 0.18 and 0.54 g/cm^3, respectively. Each gram of dry substrate produced 896 μg of total tetracycline equivalent potency. When moisture content of substrate was higher than 68%, the tetracycline production decreased. At initial moisture content 80%, total tetracycline equivalent potency was only 525 μg/g dry substrate, because packing the substrate prevented the gas exchange.

Initial pH

Tetracycline production at different initial substrate pH is shown in Figure 2. The optimal pH for tetracycline production was the original pH of sweet potato residue solid substrate, between pH 5.8 and 6.0. Each gram of dry substrate produced 1570 μg total tetracycline equivalent potency. When the initial pH was lower than 5.0, tetracycline production was low, and each gram of dry substrate produced less than 160 μg total tetracycline equivalent potency; while the initial substrate pH was higher than 6.5, tetracycline production decreased slightly. At initial pH 8.3, each gram of dry substrate produced 1000 μg of total tetracycline equivalent potency. During the fermentation, the pH change of substrate was not significant when the initial pH was lower than 5.0. The final pH of substrate was between 4.0 and 5.6 when the initial pH was higher than 5.8.

Incubation Temperature

The effect of incubation temperature on tetracycline production is shown in Figure 3. Tetracycline production had

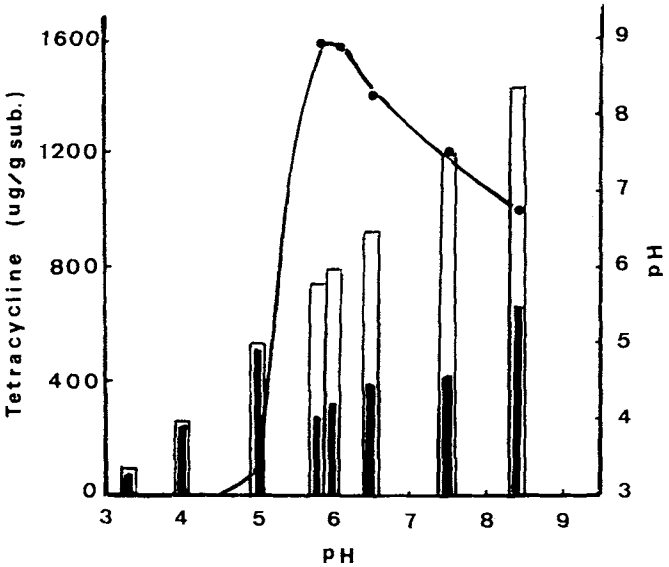

Figure 2. Effect of initial pH on tetracycline production in solid state fermentation at 26°C for five days. Culture conditions were same as Figure 1. ●—● Total tetracycline equivalent potency, ☐ Initial pH, ■ Final pH.

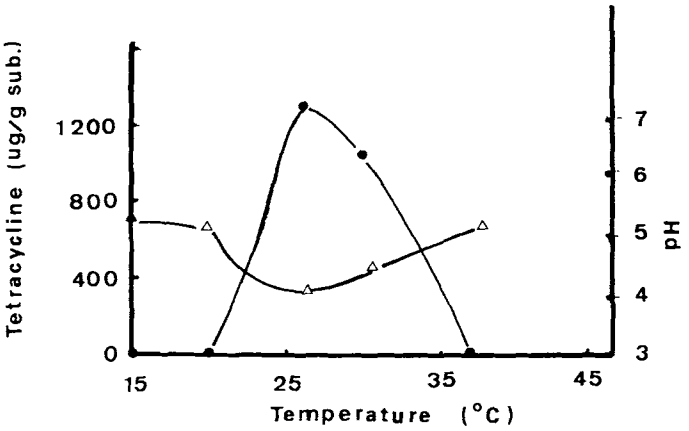

Figure 3. Effect of incubation temperature on tetracycline production in solid state fermentation. Culture media and culture conditions were same as Figure 1. ●—● Total tetracycline equivalent potency, △—△ pH.

the maximal value at 26°C, and decreased sharply when incubation temperature was higher than 37°C or less than 20°C. Each gram of dry substrate produced 1380 μg of total tetracycline equivalent potency at 26°C.

Inorganic Nitrogen Source

Sweet potato residue contained 65.4% carbohydrate and 2.32% crude protein. The C/N ratio is around 65. Therefore, nitrogen was supplemented to adjust the C/N ratio to around 20 for cell growth and tetracycline production. Effect of inorganic nitrogen source on the tetracycline production and substrate pH is shown in Table II. Although both urea and NH_4NO_3 could maintain the substrate pH, they were not suitable for tetracycline production. $(NH_4)_2SO_4$ and NH_4Cl not only resulted in appropriate substrate pH for cell growth, but also produced a high yield of tetracycline. Supplement with 0.5% (i.e., 2.4 g) $(NH_4)_2SO_4$ as nitrogen source yielded the maximum tetracycline potency. When the concentration of $(NH_4)_2SO_4$ was higher than 1%, cell growth was stimulated but tetracycline production was suppressed.

Organic Nitrogen Source

When an organic nitrogen source was used to replace the inorganic $(NH_4)_2SO_4$, soybean meal and peanut meal could enhance the production of tetracycline; while rice bran or wheat bran did not (Table II). To improve the utilization of agricultural waste, the combined nitrogen sources were studied. It showed that the mixture of organic nitrogen source and $(NH_4)_2SO_4$ had a synergistic effect on tetracycline production (Figure 4). When the mixture of 20% peanut meal and 0.5% $(NH_4)_2SO_4$ were used as combined nitrogen sources, each gram of dry substrate produced 2129 μg of total tetracycline equivalent potency. In addition, when rice bran or wheat bran was used, tetracycline production was twice that of inorganic nitrogen source only. Rice bran had a higher stimulation effect than wheat bran.

Table II. Effect of nitrogen sources on tetracycline production by solid state fermentation.[a]

Nitrogen sources (%)		Initial pH	Final pH	Bioassay method (μg/g sub.)	HPLC method (μg/g sub.) TC	CTC
None		6.08	6.44	48	6 (12.5%)	14 (29.2%)
$(NH_4)_2SO_4$	0.5%	6.04	3.82	322	34 (10.6%)	81 (25.2%)
NH_4Cl	0.5%	6.04	3.75	239	19 (8.0%)	56 (23.5%)
Urea	0.5%	6.20	6.87	24	6 (25.0%)	9 (37.5%)
NH_4NO_3	0.5%	6.11	6.80	32	19 (59.4%)	9 (28.1%)
Rice bran	20.0%	5.58	5.05	110	17 (15.5%)	16 (14.5%)
Wheat bran	20.0%	5.88	6.17	190	22 (11.6%)	35 (18.4%)
Soybean meal	20.0%	5.95	6.28	601	67 (11.1%)	142 (23.6%)
Peanut meal	20.0%	5.93	6.48	425	44 (10.4%)	90 (21.2%)
$(NH_4)_2SO_4$ 0.5% Rice bran	20.0%	5.66	3.87	1067	132 (12.4%)	329 (30.8%)
$(NH_4)_2SO_4$ 0.5% Wheat bran	20.0%	5.95	3.95	929	115 (12.3%)	283 (30.5%)
$(NH_4)_2SO_4$ 0.5% Soybean meal	20.0%	5.97	3.99	755	92 (12.2%)	199 (26.4%)
$(NH_4)_2SO_4$ 0.5% Peanut meal	20.0%	5.83	3.94	2129	389 (18.3%)	821 (38.6%)

[a] Sweet potato residue 100 g, $CaCO_3$ 1 g, NaCl 0.2 g, and 5×10^9 conidia were mixed thoroughly, and the initial moisture content was adjusted with distilled water to 68%. The substrate was incubated at 26°C for five days.

The effect of nitrogen source on the biosynthesis of tetracyclines was studied by HPLC. The ratio of these two antibiotics was dependent on the medium components and culture conditions. When NH_4NO_3 was used as the sole nitrogen source, tetracycline and chlortetracycline were 59.4% and 28.1%, respectively; while peanut meal and $(NH_4)_2SO_4$ were used as the combined nitrogen sources, tetracycline and chlortetracycline were 18.3% and 38.6%, respectively (Table II).

Inoculum Size

Each gram of dry substrate inoculated with 1.0×10^8 conidia could get 1691 μg total tetracycline equivalent potency, when the inoculum size was 1.0×10^9 (510 μg of total tetracycline equivalent potency) or less than 1.0×10^7 (<50 μg of total tetracycline equivalent potency) conidia, tetracycline production decreased in 5-days incubation.

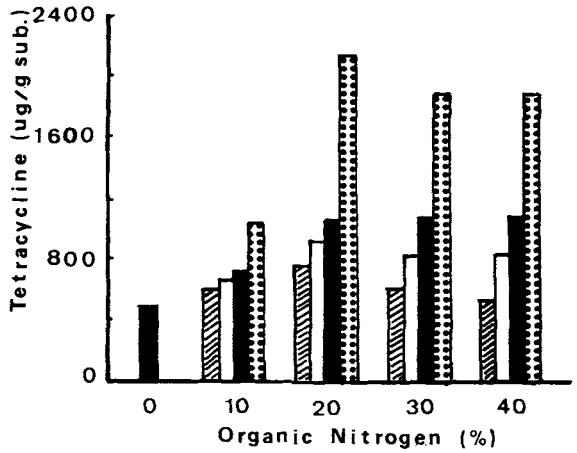

Figure 4. Effect of combined nitrogen sources on tetracycline production in solid state fermentation at 26°C for five days. Basal media and culture conditions were same as Table II. ■ Rice bran, □ Wheat bran, //// Soybean meal, ●●●● Peanut meal.

Additional Carbon Source

Effect of additional carbon source on the tetracycline production is shown in Table III. Maltose and soluble starch stimulated 2.24–2.69 times of tetracycline production; sucrose stimulated 11.2%; while glucose and galactose inhibited 60.2–68.2%.

Precursor

Effect of some precursors on tetracycline production was studied. It was found that 0.5% sodium acetate or 2.0% quinic acid inhibited tetracycline production completely; while 1.6% sodium glutamate stimulated 99%, 0.8% histidine stimulated 90% and 0.2% methionine increased two folds of tetracycline production (Figure 5).

Table III. Effect of additional carbon source on tetracycline production in solid state fermentation.[a]

Carbon source		Initial pH	Final pH	Total tetracycline equivalent potency (μg/g sub.)
None		6.08	3.86	337
Glucose	10%	5.94	3.87	107
Galactose	10%	5.95	4.05	134
Sucrose	10%	5.97	3.88	379
Maltose	10%	5.96	3.85	755
Soluble starch	10%	5.91	3.81	908

[a] Sweet potato residue 100 g, $(NH_4)_2SO_4$ 2.4 g, $CaCO_3$ 1 g, NaCl 0.2 g, and 1×10^{10} conidia were mixed thoroughly, and the initial moisture content was adjusted with distilled to 72%. The substrate was incubated at 26°C for five days.

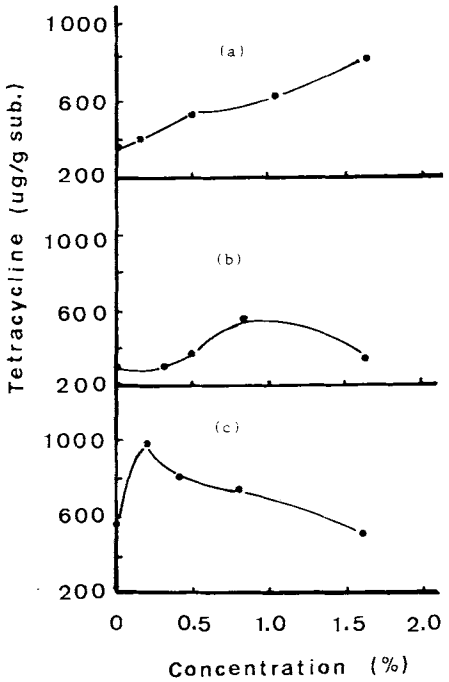

Figure 5. Effect of precursor on tetracycline production in solid state fermentation at 26°C for five days. (a) Sodium glutamate, (b) Histidine, and (c) Methionine.

Inorganic Salts

Supplement of inorganic salts could improve the production of tetracycline. 0.6–1.8% $CaCO_3$ regulated the substrate pH and stimulated tetracycline production during the fermentation (Fig. 6). 0.4% KH_2PO_4 had 11% stimulation, 0.6% NaCl had 25% stimulation, 0.8% $MgCl_2$ or 3.0% $MgSO_4 \cdot 7H_2O$ stimulated 120%. Therefore, it seems that inorganic salts were required for tetracycline production in solid state fermentation.

Extraction of Tetracycline

Solid substrate was extracted with 5-times the volume of distilled water for different shaking periods at room temperature. It was found that five minutes of shaking was enough for tetracycline extraction. Prolong shaking treatment could not improve the efficiency of extraction, but made the filtration or centrifugation troublesome. The effect of extraction volume on the recovery of tetracycline was also studied. It showed that 4–5-times the volume of distilled water was efficient for tetracycline recovery.

The above results showed that the optimum conditions for tetracycline production in *S. viridifaciens* with sweet potato residue by solid state fermentation were sweet potato residue at initial moisture content 68–72%, initial pH 5.8–6.0, supplement with $(NH_4)_2SO_4$ 0.5%, $CaCO_3$ 1.8%, NaCl 0.6%, KH_2PO_4 0.4%, $MgCl_2$ 0.8%, methionine 0.2%, sodium glutamate 0.4% and incubated at 26°C for five days. Each gram of dry substrate produced 2129 μg of tetracycline. In the case of peanut meal as the nitrogen source, the optimal conditions for tetracycline

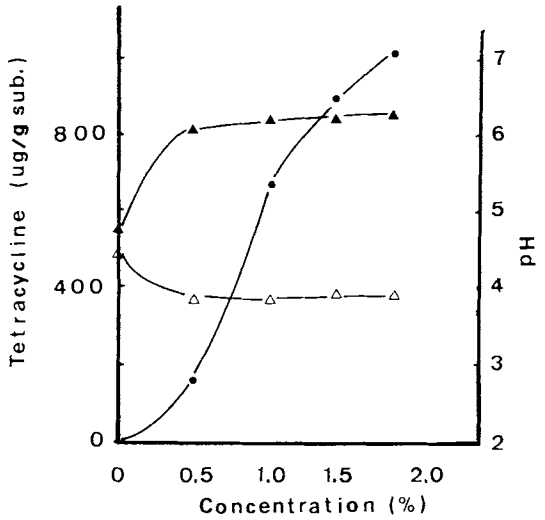

Figure 6. Effect of calcium carbonate on tetracycline production in solid state fermentation at 26°C for five days. ●—● Total tetracycline equivalent potency, ▲—▲ Initial pH, △—△ Final pH.

production were sweet potato residue supplement with peanut meal 20%, $(NH_4)_2SO_4$ 0.5%, $CaCO_3$ 1.8%, NaCl 0.6%, KH_2PO_4 0.4%, $MgCl_2$ 0.8%, soluble starch 10%, methionine 0.2%, histidine 0.8%, sodium glutamate 1.6% and other conditions were same as the above one. Each gram of dry substrate produced 4720 μg of tetracycline (Table IV).

DISCUSSION

At least 70 of the approximately 100 marketed antibiotics used for the treatment of infections in humans are derived from substances produced by *Streptomyces*.[16] Tetracyclines are broad-spectrum antibiotics used in a variety of infections caused by gram-positive and gram-negative bacteria, various rickettsias, trachoma, coccidia, amoebae, balantidia, and mycoplasma.[17] They inhibit the formation of necessary complex of ribosome, mRNA and aminoacyl-tRNA.[17]

Tetracycline was detected at the third day incubation, had maximal activity at 5–6 days, and decreased gradually in 1-month period. Tetracycline in *S. viridifaciens* was a secondary metabolite, synthesized and secreted in the late log phase or in the stationary phase.[18] In solid state fermentation, the mechanism of tetracycline secretion was same as that proposed in submerged fermentation. However, in submerged fermentation, antibiotic activity decreased sharply after prolonged incubation due to cell autolysis.[18] Therefore, antibiotic production by solid state fermentation was more stable than that in submerged fermentation and the product could be temporarily stored without losing activity significantly.[8]

Water, especially the initial moisture content of substrate, was very important in the solid state fermentation.[19] The initial moisture content of substrate for maximal tetracycline production was at 68–72%, and the final moisture content was 70–74%. During the fermentation, moisture

Table IV. Effect of ingredients on tetracycline production in solid state fermentation with initial moisture content 68% at 26°C for five days.

Medium Item		C-0	C-1	M-2	M-3	M-4	R-5	R-6	R-7	R-8	R-9	P-10	P-11	P-12
Sweet potato residue	(g)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
$(NH_4)_2SO_4$	(%)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
$CaCO_3$	(%)	1.0	1.8	1.8	2.5	3.6	1.0	1.8	2.5	3.6	1.8	1.0	1.8	1.8
NaCl	(%)	0.2	0.6	0.6	0.6	0.6	0.2	0.6	0.6	0.6	0.6	0.2	0.6	0.6
KH_2PO_4	(%)	0.0	0.4	0.4	0.4	0.4	0.0	0.4	0.4	0.4	0.4	0.0	0.4	0.4
$MgCl_2$	(%)	0.0	0.8	0.8	0.8	0.8	0.0	0.8	0.8	0.8	0.8	0.0	0.8	0.8
Methionine	(%)	0.0	0.0	0.2	0.2	0.2	0.0	0.2	0.2	0.2	0.2	0.0	0.2	0.2
Histidine	(%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0	0.8
Sodium glutamate	(%)	0.0	0.0	0.4	0.4	0.4	0.0	0.4	0.4	0.4	1.6	0.0	0.4	1.6
Soluble starch	(%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0	0.0	0.0	10.0
Rice bran	(%)	0.0	0.0	0.0	0.0	0.0	20.0	20.0	20.0	20.0	20.0	0.0	0.0	0.0
Peanut meal	(%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	20.0	20.0	20.0
Initial pH		6.04	5.80	5.85	6.00	6.10	5.66	5.60	5.65	5.70	5.80	5.83	5.80	5.80
Final pH		3.82	4.15	4.13	4.87	6.48	3.87	4.76	5.10	5.82	4.95	3.94	4.87	4.90
Total tetracycline equivalent potency (μg/g sub.)		600	1525	2129	1067	755	1067	2281	673	379	2890	2129	3964	4720

content of substrate increased, which might be due to the production of metabolic water of *Streptomyces*. Similar results have been obtained with sugar beet residue,[20] sweet potato residue[12] for protein enrichment, sweet potato residue for protease production,[11] and compost preparation.[19]

Substrate pH decreased gradually in the first five day incubation. Matsushima et al.[21] indicated that the pH drop in the acid protease production might be due to the accumulation of organic acid and the residue of sulfate ion in the utilization of $(NH_4)_2SO_4$. Therefore, the pH of substrate might be maintained by a supplement with alkali,[22] or appropriate salts,[23] or other kinds of nitrogen sources.[11,12,24,25] In tetracycline production, when the initial substrate pH was lower than 3.3, it was unfavorable to antibiotic production. Calcium carbonate was frequently added to the medium to counteract excess acidity and to enhance the tetracycline production, but an abundance might interference in the extraction procedure.[11]

The optimal temperature for tetracycline production was dependent on the tested organisms.[4] In this study, *S. viridifaciens* had an optimal temperature of 26°C for tetracycline production in solid state fermentation. Nitrogen supplement could reduce the C/N ratio and enhance the cell growth and antibiotic production. $(NH_4)_2SO_4$ was the best inorganic nitrogen source and peanut meal, rice bran, wheat bran, and soybean meal were the candidate organic nitrogen sources in tetracycline production. This result was consistent with Wang's result in chlortetracycline production with *S. aureofaciens*.[7] In addition, combined nitrogen sources could enhance the tetracycline production over that of organic or inorganic nitrogen alone. The ratio of tetracycline and chlortetracycline production was also affected by the nitrogen source supplement. $(NH_4)_2SO_4$ and NH_4Cl favored chlortetracycline production, while NH_4NO_3, urea, organic nitrogen sources, and combined nitrogen sources favored the tetracycline production.

Each gram of dry weight substrate inoculated with 1.0×10^8 conidia could produce 1691 μg of total tetracycline equivalent potency. When the inoculum size was either 1.0×10^9 or less than 1.0×10^7 conidia, the total tetracycline equivalent potency decreased sharply. The optimum inoculum cell size in protein enrichment in solid state fermentation was 2.6×10^7 conidia of *Aspergillus niger* per gram of substrate,[24] or 8.0×10^7–1.0×10^8 cells of yeasts.[12]

Monosaccharide was good for cell growth, but inhibited the production of secondary metabolites. Disaccharide and polysaccharide slightly stimulated secondary metabolite production. Vlasta et al. reported the same phenomenon in oxytetracycline production with *S. rimosus*.[26] Therefore, supplement with a small amount of soluble starch or other fermentable polysaccharide might be good for secondary metabolites production.

Gourevitch and Lein indicated that quinic acid, shikimic acid, and related substances would stimulate the production of tetracycline.[4] Miller et al. showed that acetate was the precursor of tetracycline, while addition of shikimic acid could not improve the biosynthesis of chlortetracycline.[27] Snell et al. indicated that glutamate was the precursor of oxytetracycline, and provided the 2-carboxyl and 4-amino group in C2–C4a of A-ring.[28] The biosynthesis of antibiotic was connected with the carbon and lipid metabolism. This study showed that quinic acid and acetic acid inhibited the biosynthesis of tetracycline. The addition of L-methionine and L-histidine improved 3 times the 7-chlortetracycline production.[8] In this study, the same phenomenon was found in tetracycline production.

Niedercorn showed that salts of calcium, magnesium or both not only adjusted the pH of substrate, but also decreased the toxicity of the antibiotic to the tested organisms,[29] while Borenstaju and Wolf[30] and Nakayama[31] reported that phosphate salts would inhibit the tetracycline,

chlortetracycline and oxytetracycline production in submerged fermentation. In this study, it was found that $CaCO_3$ and NaCl would stimulate the tetracycline production. Wang indicated that chlortetracycline production was connected with the uptake of chloride ion.[7] Chlortetracycline concentration increased with the concentration of sodium chloride from 0.2% to 0.8%.[8]

In the submerged culture, each mL of culture broth produced more than 12 mg of tetracycline or chlortetracycline when the medium contained sucrose, soybean meal, cornsteep liquid and inorganic salts.[31] In this study, although each gram of substrate produced 4.72 mg of tetracycline, the product was more stable than that in submerged culture, and the energy input was also less than that in submerged culture.[32,33] Therefore, production of antibiotics with solid state fermentation might be a feasible process in agricultural waste treatment and utilization.

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