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Bioconversion of Aniline to Acetaminophen and Overproduction of Acetaminophen by *Streptomyces* spp.

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Abstract □ In order to obtain acetaminophen, a popular analgesic-antipyretic, though microbial p-hydroxylation and N-acetylation of aniline, various *Streptomyces* strains were screened. Aniline N-acetylation activity was rather ubiquitous but p-hydroxylation activity was selective. Microbial conversion pathway of aniline to acetaminophen was considered to be through N-acetylation and p-hydroxylation or *vice versa*. However, depending on species used, o-hydroxylation and its degradation activity (*S. fradiae*) and acetaminophen degradation activity (*S. coelicolar*) were also detected. Among the screened *Streptomyces* strains, *S. fradiae* NRRL 2702 showed the highest acetanilide p-hydroxylation activity (2-3% conversion rate). Furthermore, in *S. fradiae* carbon source and its concentration, phosphate ion concentration and pH of growth medium were found to play the crucial roles in p-hydroxylation activity. Through the proper combination of factors mentioned above, the ten times more activity (26-30% conversion rate) was attained.

Keywords □ Aniline, acetaminophen, hydroxylation, *Streptomyces fradiae*, acetaminophen degradation activity, medium component effect, high production

As a popular analgesic and antipyretic, acetaminophen lacks some of the side effects of aspirin, and has been used as the largest aspirin substitute. Microbial transformation of aniline to acetaminophen was interested for performing concomitant N-acetylation and p-hydroxylation in one reaction vessel and its regiospecific monooxygenation to aromatic ring similar to that observed in mammalian metabolism. In general, microorganisms can degrade aromatic compounds, use them as a carbon source and this degradation pathway includes catechol formation by dioxygenase[1]. However, we cannot exclude the possibility of the formation of phenolic compound through the action of monooxygenase. *Cunninghamella bainieri* and *Bacillus megaterium* ATCC 13368 were found to hydroxylate aniline to p-aminophenol[2,3]. Aniline is also actively converted to 2-hydroxyacetanilide (2-acetamidophenol) by *Aspergillus ochraceous* ATCC 1008. This microorganism accumulates large amouts of 2-hydroxyacetanilide when acetanilide was used as the substrate. Because N-acetylation of aniline is rather ubiquitous in *Streptomyces* species, aniline is apt to be converted to acetanilide. Furthermore, unidentified *Streptomyces* species were reported to convert acetanilide to 2′-hydroxyacetanilide and 4′-hydroxyacetanilide (4-acetamidophenol; acetaminophen) with 4-hydroxy isomer predominating[6]. In general, regulation of biosynthesis by readily used sources of carbon, nitrogen and phosphorus affects the production of cells for bioconversion[7]. And bacterial P450-dependent hydroxylases can be induced by substrate, environmental factors and non-substrate substances[8]. Most bacterial P450 dependent hydroxylases are induced by substrates[9]. However, non-substrate and environmental factors inducing P450 are relatively uncommon in bacteria. Only two examples are known: the induction of $P450_{soy}$ in *S. griseus* by the isoflavonoid, genistein[10], and the induction of three P450s in *Bacillus megaterium* by barbiturate[11]. In this study, we elucidated some aspects of acetaminophen synthetic pathway, selected acetaminophen high production microorganism among the *Streptomyces* species screened and composed proper media for acetaminophen production.

Table I. Comparison of acetanilide p-hydroxylation activity in *Streptomyces* species

Streptomyces spp.		p-hydroxylation activity
S. lividans	ACTC 1159	++(8.6 μg/m*l*)
S. lividans	ACTC 1163	+(2.1 μg/m*l*)
S. lividans	ACTC 1167	+
S. fradiae	NRRL 2702	++(16 μg/m*l*)
S. fradiae	IFO 3718	−
S. fradiae	NRRL 1195	+−(+)
S. fradiae	NRRL 3357	+(<5 μg/m*l*)
S. aureofaciens	ATCC 10762	+(<5 μg/m*l*)
S. globisporus	ATCC 1829	+
S. espinosus	NRRL 11439	+−(+)
S. kanamyceticus	NRRL 2135	−
S. kanamyceticus	IFO 13414	−
S. rimosus	NRRL 2234	+
S. rimosus paromomycinus	NRRL 2445	+−
S. griseus	NRRL 3851	+−
S. kitasatoensis	NRRL 2486	+−
S. coelicolor	A3 (2)	+

−, negative p-hydroxylation activity
+−, very weak p-hydroxylation activity
+, positive but weak p-hydroxylation activity
++, strong p-hydroxylation activity

EXPERIMENTAL METHODS

Cultures

The *Streptomyces* strains used in this study are listed in Table I. The organisms were maintained on Sporulation agar slant medium (ATCC medium 5), and were stored in a refrigerator at 4℃ prior to use.

Culture conditions

Cultures were all grown in Theriault's screening medium of the following compositions: glucose 50g; soybean flour 5g; yeast extract 5g; KH_2PO_4 1g; K_2HPO_4 2g; NaCl 5g; distilled water 1000 m*l*; adjusted to pH 7.0. Media were sterilized in an autoclave at 121℃ for 15 min before use. The surface growth from slants of microorganisms was used to inoculated 1 m*l* of Theriault's screening medium held in cotton-plugged 10 m*l* Erlenmeyer flasks. The flasks were incubated at 28℃ on a rotary shaker operating at 180 rpm until full mycelial mass developed (usually more than 48 hrs). The cultures were transferred (10%, v/v) to fresh Theriault's screening medium held in cotton-plugged 10 m*l* Erlenmyer flasks. After 48 hrs of incubation on the rotary shaker, saturated acetanilide aqueous solution (5 mg/m*l*) was added to the final concentration of 0.5 mg/m*l*. The substrate-containing flasks were incubated for an additional 5 days or more before being terminated. All cultures were fully grown when fermentations were terminated.

Sampling procedures

In the standard procedure, samples of 5 m*l* were withdrawn after termination of fermentation, adjusted to pH 1 with dilute HCl, and extracted with equal volume of ethylacetate three times. The pooled ethylacetate extracts were evaporated to dryness under vacuum or on a water bath. The residues were dissolved with 50 μ*l* methanol. But for colorimetric analysis, 0.2 m*l* of culture broth was withdrawn.

Methods of analysis

Acetaminophen formed was determined by TLC, colorimetry and HPLC. For TLC, approximately 10 μ*l* of methanol extracts were spotted on TLC plates. Reference compounds and extracts of incubation mixtures were chromatographed 10 cm on 250 μm silica gel GF_{254} layers in the following solvent system: $CHCl_3/CH_3OH/NH_4OH$ (85:15:1.5). Products were identified by comparison of R_f values. Spot locations were confirmed by quenching of 254 nm induced fluorescence and/or sparying the solution of 1% $K_3Fe(CN)_6$ and $FeCl_3$ (1:1, v/v)[12], determined on separate TLC layers. For product isolation, column chromatography was carried out by changing the eluting solvent linearly from $CHCl_3$:CH_4OH:NH_4OH=85:15:1.5 to 85:15:3. The product was recrystalized from hot acetone and analyzed. Estimation of conversion to acetaminophen was carried out according to the procedure of Shihabi and David[13] with slight modification. After centrifugation (5,000×g, 10 min) of culture, 0.2 m*l* of supernatant was removed and mixed with 2-nitroso-1-naphthol-4-sulfonic acid (NNS) and trichloroacetic acid solutions (final concentrations, 5g/*l* and 100g/*l* respectively). The mixture was centrifuged (4,000×g, 20 min) to remove the protein precipitate. For full color development, 0.2 m*l* of

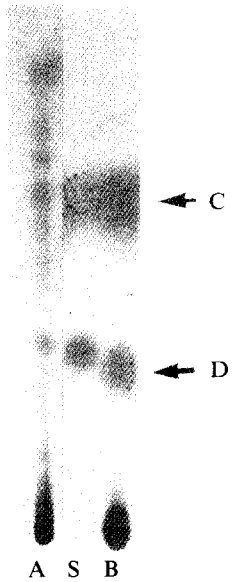


Fig. 1. Degradation of acetanilide and acetaminophen by *S. coelicolor* A3 (2).
Lane A, metabolites in culture of 10 day incubation; lane B, metabolites in culture of 5 day incubation; lane S, standards; C and D represent acetanilide and acetaminophen.

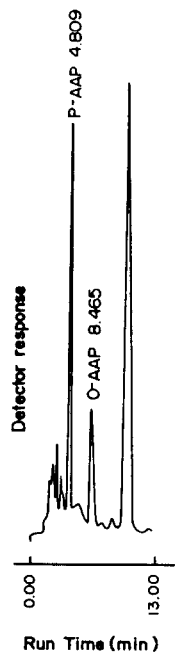


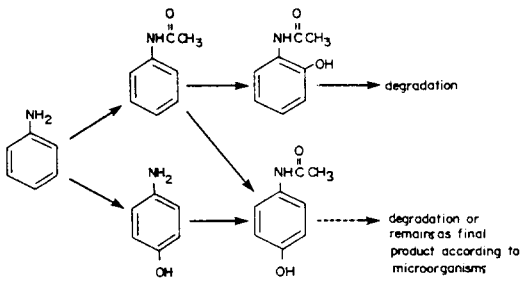
Fig. 2. Separation of 2-acetamidophenol and 4-acetamidophenol in *S. fradiae* culture broth by HPLC.

$NaNO_2$ solution (1g/*l*) was added to 1.8 m*l* of supernatant, and allowed to stand for 20 min. And then optical density was determined at 530 nm against a blank (culture broth in the absence of substrate) in the reference beam. HPLC was done with acetonitrile/1% acetic acid in water (15:85, v/v), on a μBonndapak C 18 column (30 cm×4 mm) at a flow rate of 1.1 m*l*/min. Injection volume was 10 μ*l* and 280 nm UV absorbance detector was used.

RESULTS AND DISCUSSION

Bioconversion of aniline and acetanilide by Streptomyces spp.

The hydroxylase activities of *Streptomyces* species tested were detected primarily using thin layer chromatography technique. And then conversion to acetaminophen was estimated quantitively with colorimetric method. This colorimetric method was originally developed for determination of acetaminophen (4-acetamidophenol) in serum, based on its reaction with 2-nitroso-1-naphthol-4-sulfonic acid[13]. It was reported that acetaminophen metabolites and other structurally similar drugs did not cause any interference in the determination of acetaminophen. This method could be applied to microbial fermentation product successfully with slight modification. However, different from *in vivo* mammalian metabolism, microorganism could produce some other metabolites. To test the interferences from possible microbial metabolites, five compounds (2-aminophenol, 3-aminophenol, 4-aminophenol, 2-acetamidophenol and 3-acetamidophenol) were reacted with NNS and $NaNO_2$. The absorption maximum of acetaminophen is at 530 nm (red color), with a relative molar absorptivity of 19,170 2-Acetamidophenol besides acetaminophen developed red color with color reagents (relative molar absorptivity at 530 nm: 8,780). But the amount of 2-acetamidophenol produced in culture was below 25 μg/m*l*. In addition, 2-acetamidophenol was degraded soon and disappeared when the production of acetaminophen was maximum. To determine these substances separately, analysis with HPLC was carried out. The relative acetanilide p-hydroxylation activities of *Streptomyces* spp. are indicated in Table I. Among microorganisms utilized, *S. coelicolor* showed the highest hydroxylation activity at beginning. When it was grown in modified Theriault's medium (described



Scheme 1. Microbial conversion pathway of aniline and acetanilide in *Streptomyces*.

below), *S. coelicolor* formed large amount of acetaminophen (200g/*l*) in the early fermentation period but degraded it rapidly (Fig. 1). On the other hand, *S. fradiae* accumulated acetaminophen rather slower but in the resonable amounts (15-20g/*l* from 500g of aniline hydrochloride or acetanilide per liter) representing 3-4% bioconversion in Theriault's medium (see below). Acetanilide is principally converted to 2-acetamidophenol by sevral microorganisms[4]. Theriault and Longfield also found six *Streptomyces* species which hydroxylated acetanilide in the 2- and 4-positions with 4-hydroxy-isomer predominating. Acetanilide was hydroxylated to 2-acetamidophenol by *S. fradiae* as well. This substance was separated by column chromatography and recrystalized from hot acetone. The major product isolated as 2-acetamidophenol was identical to authentic 2-acetamidophenol by UV and IR spectrum and HPLC retention time (Fig. 2). The accumulations of 2-acetamidophenol and 4-acetamidophenol were investigated with time. 4-Acetamidophenol was found to accumulate in culture broth with time but 2-acetamidophenol was degraded gradually (Fig. 3). Acetaminophen degradation activity was not also observed in *S. aureofaciens*. Mutants selected among the colonies of regenerated *S. fradiae* protoplasts showed the enhanced o-hydroxylation activity and smaller amounts of acetanilide remained. Acetaminophen can be obtained from aniline through microbial p-hydroxylation and N-acetylation[5], but according to strains used 2-acetamidophenol could be formed and 2-acetamidophenol and 4-acetamidophenol could be degraded (Scheme 1).

Medium components influencing hydroxylation activity

Theriault's screening medium had been developed

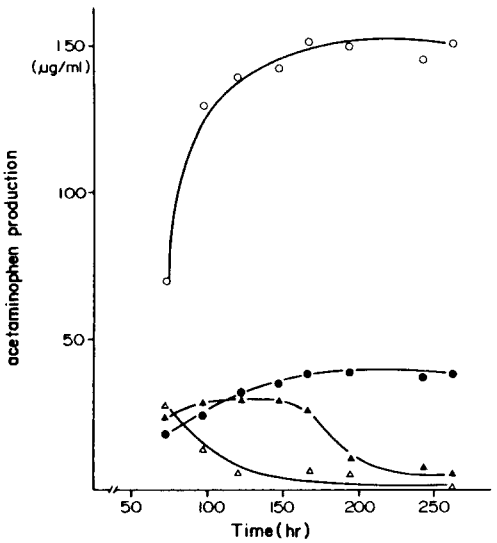


Fig. 3. Time courses of formation of 2-acetamidophenol and 4-acetamidophenol by *S. fradiae* wild type and mutant in modified Theriault's screening medium. Production of 2-acetamidophenol and 4-acetamidophenol was determined by HPLC. ○, 4-acetamidophenol formed by wild type; △, 2-acetamidophenol formed by wild type; ●, 4-acetamidophenol formed by mutant; ▲, 2-acetamidophenol formed by mutant.

for the medium in which microbial transformation of acetanilide to acetaminophen was carried out[6]. Theriault and Longfield obtained four times more acetaminophen through changing the commposition of medium using taxonomically unchacterized *Streptomyces* strain. Furthermore, bacterial P-450-dependent hydroxylases can be induced by substrate, environmental factors and non-substrate substances. The $P450_{soy}$ in *S. griseus* are known to be induced by culture medium component isoflavonoid, genistein[10]. During the course of investigation on medium component influencing on acetanilide p-hydroxylation activity in *S. fradiae*, yield improvement of acetaminophen was observed in Theriault's medium deprived of KH_2PO_4. Another increased yield was obtained by substitution of sucrose for glucose (Fig. 4). These facts imply that adjustment of phosphate ion cocentration and pH and proper choice of carbon source can lead to increased acetaminophen formation by *S. fradiae*. In contrast, the p-hydroxylation activity in *S. aureofaciens* was not indu-

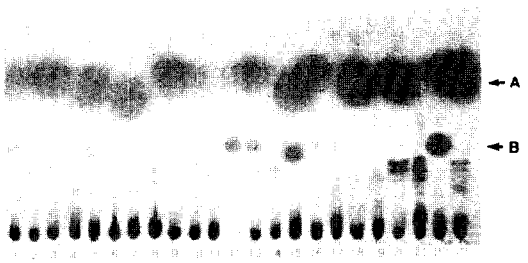


Fig. 4. Influence of components of Theriault's screening medium on bioconversion of acetanilide to acetaminophen by *S. aureofaciens* and *S. fradiae*.
Lane 1-11, *S. aureofaciens*; lane 12, standards; lane 13-23, *S. fradiae*; lane 1, 23, without K_2HPO_4; lane 2, 22, without KH_2PO_4; lane 3, 18, without glucose; lane 4, 19, blank without glucose; lane 5, 16, without yeast extract; lane 6, 17, blank without yeast extract; lane 7, 20, without phosphate ion; lane 8, 21, blank without phosphate ion; lane 9, 15, without sucrose as carbon source; lane 10, 13, positive control in normal Theriault's screening medium; lane 11, 14, negative control without acetanilide in Theriault's screening medium; A, acetanilide; B, acetaminophen.

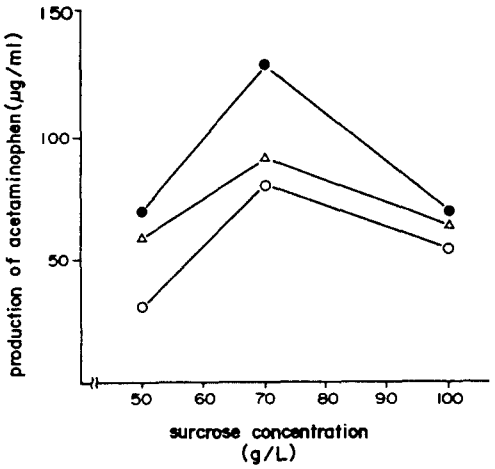


Fig. 5. Effect of concentration of sucrose (g/l) on bioconversion of acetanilide to acetaminophen.
△, medium containing 3g/l of phosphate ion, pH 8.0; ●, medium containing 4g/l of phosphate ion, pH 8.0; ○, medium containing 5g/l of phosphate ion, pH 8.0.

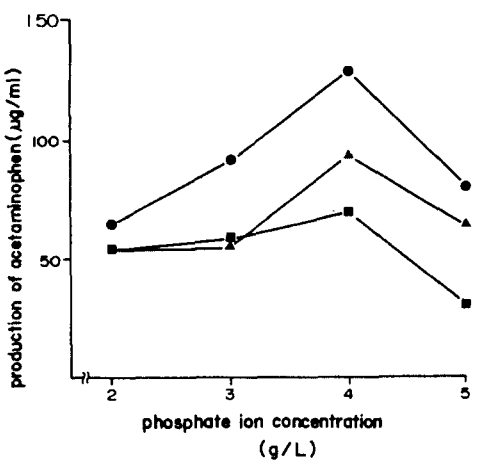


Fig. 6. Effect of concentration of phosphate ion (g/l) on bioconversion of acetanilide to acetaminophen.
●, medium containing sucrose of 70g/l, pH 8.0; ■, medium containing sucrose of 50g/l, pH 8.0; ▲, medium containing of sucrose 50g/l, pH 7.7.

ced or affected apparently by the medium component (Fig. 4). The conversion rate was proportional to cell growth apparently. Effects of pH and phosphate ion concentration were investigated through preparing the appropriate combination of KH_2PO_4, K_2HPO_4 and K_3PO_4 (ranges of phosphate ion concentration and pH were 2-5g/l and 6.5-8.0 respectively. To select the proper carbon source, sucrose, fructose, galactose, lactose and glycerol were tested. The substitution of sucrose and fructose for glucose gave the increased yield of acetaminophen among the carbon sources used at the concentration of 50g/l. In case of sucrose, the conversion rate was doubled than glucose and fructose and the amount of acetaminophen produced per unit cell mass was increased to 1.2 times. Increase of sucrose concentration from 50g/l to 70g/l gave the improved yield of acetaminophen but higher concentration (100g/l) led to the reduced yield and production per unit cell mass (Fig. 5). Cell growth was inhibited with higher concentration of fructose and at the same time acetaminophen product ion was reduced. Higher (5g/l) and lower (2g/l) concentrations of phosphate ion showed the reduced acetaminophen formation and growth. The proper concentrations were 3g/l for glucose and 4g/l for sucrose and fructose (Fig. 6). The cell growth and acetaminoplen production were diminished below pH 7. Optimum pHs were pH 7.4-7.7 for glucose and pH 7.7-8.0 for sucrose and fructose (Fig. 7). In each optimum pH, yield per unit cell mass was also increased. Increased carbon source concentration and high phos-

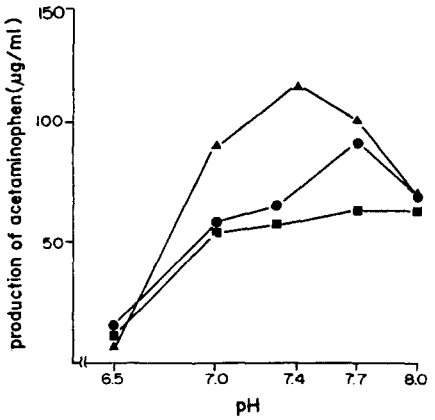


Fig. 7. Effect of initial pH of medium on bioconversion of acetanilide to acetaminophen.
▲, medium containing 50g/*l* of glucose, 3g/*l* of phosphate ion; ●, medium containing 50g/*l* of sucrose, 3g/*l* of phosphate ion; ■, medium containing 70g/*l* of fructose, 4g/*l* of phosphate ion.

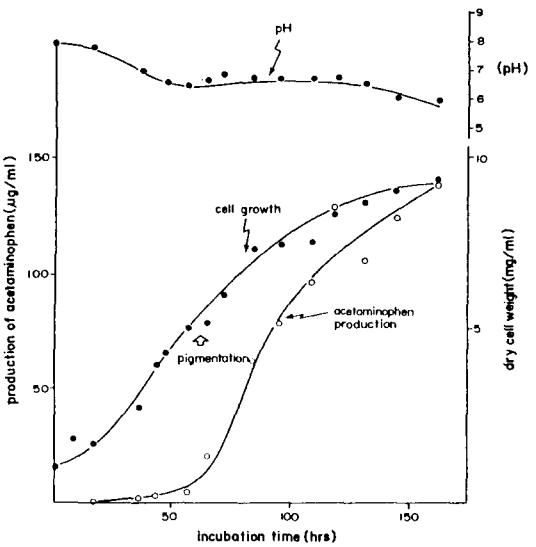


Fig. 8. Cell growth, production of acetaminophen and change of pH during the bioconversion of acetanilide by *S. fradiae* in modified Theriault's screening medium.
Acetaminophen production was increased sharply after pigmentation of mycelia.

phate ion concentration did not affect the amount of acetaminophen produced per unit cell mass but owing to the reduced cell growth, total amount of acetaminophen was decreased. Through putting the factors mentioned above together, the most optimum composition of medium (modified Theriault's medium, sucrose 70g/*l*, soybean flour 5g/*l*, yeast extract 5g/*l*, NaCl 5g/*l*, K_2HPO_4 2.95g/*l*, K_3PO_4 1.62g/*l*) was obtained that is, 70g/*l* of sucrose, 4g/*l* of phosphate ion and pH 8.0. However, the induction effect of substrate, acetanilide was not observed.

Growth and overproduction of acetaminophen

The modified Theriault's medium was inoculated with *S. fradiae* mycelia and acetaminophen formation was investigated with time course of fermentation. Fig. 8 shows the growth and acetaminophen accumulation observed upon adding acetanilide to a culture just started fermentation at the concentration of 500g/*l*. The first acetaminophen formation was detected 19 to 20 hrs after inoculation (early exponential phase) by TLC. Interestingly, it was also found that accumulation rate was sharply increased just after red brownish coloration of mycelia. Moreover, two acetaminophen non-producing mutants obtained through protoplast formation and regeneration had the same morphological characteristics and cell growth as wild type but coloration of mycelium did not occur. These results indicate that two processes are intimately coupled. Maximal yield of 130-150g/*l* of acetaminophen (26-30% conversion rate), 10 times increased conversion than Theriault's screening medium, was attained 150 hrs after incubation (stationary phase).

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