0273-2289/97/63–65—0707$10.75

The Degradation of L-Tyrosine to Phenol and Benzoate in Pig Manure

The Role of 4-Hydroxy-Benzoate

P. ANTOINE,*[,1] X. TAILLIEU,[1] AND P. THONART[1,2]

[1]*Centre Wallon de Biologie Industrielle, Faculté des Sciences Agronomiques, 2, Passage des Déportés B-5030 Gembloux, Belgium; and* [2]*Université de Liège, Bâtiment 40, Boulevard du Rectorat, B-4000, Sart-Tilman, Belgium*

ABSTRACT

The formation of odorous compounds in piggery wastes was investigated. Phenol and *para*-cresol are generally encountered in these typically anaerobic environments. They are produced from L-tyrosine by microbial metabolism. Phenol is further converted to benzoate via *para*-carboxylation.

The biochemical pathways were studied by feeding manure with miscelleanous metabolites at concentration between 5 and 20 m*M*. Metabolites were analyzed by gas chromatography (GC) and high-performance liquid chromatography (HPLC). Experiments were carried out at room temperature.

The degradation of L-tyrosine to phenol, benzoate, and *para*-cresol was confirmed. 4HPPyrA and 4HPAA are not intermediate compounds in phenol production.

It was shown that phenol was converted to benzoate without any production of 4HBA. Other experiments showed that 4HBA was decarboxylated to phenol, but not dehydroxylated to benzoate.

When phenol was added in presence of benzoate (5 m*M* each) or alone at higher concentrations (10 or 20 m*M*), transient small amounts of 4HBA were observed (about 0.02 m*M*).

Our experiments show that 4HBA is not an intermediate metabolite in the conversion of phenol to benzoate. The decarboxylation of 4HBA to phenol is probably the last step of another degradation pathway. This reaction is proposed to have a weakly reversible property, explaining 4HBA production.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: L-tyrosine; benzoate; 4-hydroxybenzoate; phenol; degradation; piggery; anaerobic.

Abbreviations: 4HBA, 4-hydroxybenzoate; 4HPPyrA, 4-hydroxyphenylpyruvate; 4HPAA, 4-hydroxyphenylacetate.

INTRODUCTION

The intensive breeding of pigs leads to the accumulation of large amounts of malodorous and polluting slurries. These wastes are mainly constitued of feces and urine.

Schaefer identified 10 molecules as important factors of malodor in piggeries: indole, skatole, phenol, *para*-cresol, acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate *(1)*.

Phenol is a degradation product of L-tyrosine by *Clostridium* sp. *(2,3)* or other bacteria possessing a tyrosine phenol lyase activity *(4)*. This conversion has been observed in anaerobically stored piggery wastes *(4–6)*. Another precursor is 4-hydroxybenzoate (4HBA), which was reported to be decarboxylated to phenol *(7)*.

Two degradation pathways of phenol have been proposed. The first one includes cyclohexanone *(8)*. The latter is a conversion of phenol to benzoate *(9)*. Tschech and Fuchs observed that bicarbonate is necessary to ensure the carboxylation of phenol by a denitrifying Pseudomonads *(10)*. They also observed that radioactivity was transfered from $^{14}CO_2$ to 4HBA in the presence of 4HBA by phenol-grown cells *(10)*. This indicates the reversible cleavage of 4HBA to CO_2 and to enzyme-bound phenol.

Sharak-Genther et al. desmonstrated by the use of fluororophenols that benzoate was formed via a *para*-carboxylation of phenol *(11)*. Interestingly, it was shown that 3-fluoro-4-hydroxybenzoate was converted to 3-fluorobenzoate. This reaction was expected in the case of 4HBA dehydroxylation to benzoate.

Zhang and Wiegel studied the degradation of 2,4-dichlorophenol under methanogenic conditions *(12)*. They proposed that a microorganism included in their consortium was first decarboxylating 4HBA to phenol before converting phenol to benzoate. 4HBA was not detected during phenol conversion to benzoate. These authors suggest that this metabolite could only exist under a enzyme-bound form.

Béchard et al. desmonstrated that the presence of proteose peptone was necessary to observe the conversion *(13)*. This suggests the existence of a cometabolism. Conversely, the presence or absence of H_2 had no influence on the reaction. Bisaillon et al. observed that proteose peptone could be replaced by tryptophan and lysine *(14)*.

In this article, we present results concerning the degradation of L-tyrosine to phenol in piggery wastes. The conversion of phenol to benzoate was investigated with an emphasis on the role of 4HBA.

METHODS

Slurry Sampling and Storage

The slurries were collected in piggeries of Gembloux countryside, Belgium. They were collected in pits under animals. The slurries were stored at 4°C until their use for experiments.

L-Tyrosine Degradation

Three samples of slurry from different piggeries were fed with 5 m*M* of L-tyrosine. The volume of the samples was 100 mL. Phenol, *para*-cresol, and benzoate were measured during the following days by gas chromatography (GC). The piggery slurries were placed in 100-mL flasks for experiments. The flasks were kept at room temperature during assays. In all experiments, a flask of slurry without chemical added was used as control. The slurries were gently mixed before sampling. The volume of samples was about 5 mL.

The role of 4-hydroxyphenylpyruvate (4HPPyrA) and 4-hydroxyphenylacetate (4HPAA) as intermediate compounds in phenol production pathway was investigated. These metabolites were added to the slurries at the approximative concentration of 5 m*M*. Phenol concentration was measured during the following days.

Phenol, Benzoate, and 4HBA Degradation

The slurry was complemented with phenol, benzoate, or 4HBA at the approximative concentration of 5 m*M*. The concentration of these three metabolites was measured during the following days by GC or high-performance liquid chromatography (HPLC). In a second series of experiments, the slurry was complemented simultaneously by 5 m*M* of phenol and various concentrations of benzoate (0, 5, 10, or 20 m*M*. The experiments were carried out in the same conditions as those described for L-tyrosine degradation.

Adapted Slurries

A slurry in which degradation activities are already expressed is called an adapted slurry.

Gas Chromatography

Five milliliters of slurry were extracted for 2 h with 2 mL of diethyl-ether in the presence of 400 mg of NaCl and 400 mL of a 50% solution of sulfuric acid. The diethyl-ether contained 500 mg/L of dimethylmalonate as internal standard. The ether extract was dehydrated by addition of anhydrous sodium sulfate. It was then ready for GC analysis.

A Hewlett Packard 5890 series II chromatograph was used with an FFAP column and an FI detector. The column length was 25 m. Its internal

diameter was 0.32 mm. The carrier gas was nitrogen. The oven temperature was controlled to obtain the best separation of peaks. It was maintained at 34°C for 30 s, increased to 220°C (15°C/min from 34–100°C; 10°C/min from 100–160°C; 15°C/min from 160–220°C), and finally maintained at 220°C for 10 min.

Five microliters of ether extract were injected with the Hewlett Packard 7673 automatic sampler. Phenol and benzoate were measured by this method. 4HBA was not detected. Accuracy and linearity were confirmed in the concentration ranges of the experiments.

High-Performance Liquid Chromatography

One milliliter of slurry was diluted by 5 mL of distilled water. This diluted sample was centrifuged at 27,000g rpm for 10 min to eliminate the solid particules. Three milliliters of supernatant were diluted with 2 mL of acetonitrile and 1 mL of acetic acid. This solution was then filtered with a 0.45-μ filter. We used a Hewlett Packard series 1050 chromatograph equipped with a 250/8/4 Nucleosil $5C_{18}$ column. Five microliters of solution were injected with a Bio-Rad AS-48 automatic sampler. Metabolites (phenol, benzoate, and 4HBA) were eluted (flow = 4.00 mL/min) with a mixture of water, acetonitrile, and acetic acid (3:2:1). They were measured with a diode array detector near their maximum absorption wavelength (4HBA: 256 nm, phenol and benzoate: 270 nm) with a bandwidth of 4 nm. The nature of the metabolites was confimed by their absoption spectrum.

Accuracy and linearity were confirmed in the concentration ranges of the experiments.

RESULTS

Degradation of L-Tyrosine to Phenol, *Para*-cresol, and Benzoate

A transient accumulation of phenol, *para*-cresol, and benzoate was observed in the three samples of piggery wastes (A, B, and C) in which L-tyrosine had been added. This is described in Fig. 1.

The accumulation kinetics of phenol were similar in the three slurries, except that in case C, a steady phase was observed before the degradation of phenol. The average accumulation rates were 0.45, 0.50 and 0.57 mmol/L/d in the cases A, B, and C, respectively. The phenol average degradation rates were 0.28, 0.32 and 0.37 mmol/L/d, respectively. Phenol was produced in each sample in larger amounts than *para*-cresol. This is described in Table 1.

Conversion of L-Tyrosine to Phenol: the Role of 4HPPyrA and 4HPAA

The role of 4HPPyrA and HPAA as intermediate compounds in the conversion of L-tyrosine to phenol was investigated. Phenol was produced in the largest amount when L-tyrosine was used as precursor metabolite.

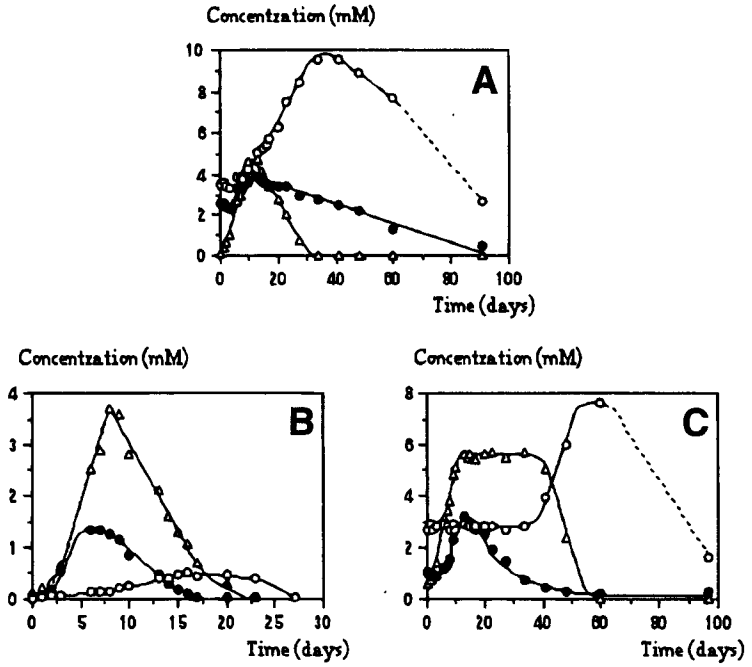

Fig. 1. Phenol (Δ), benzoate (○), and *para*-cresol (●) were measured after the addition of 5 m*M* of L-tyrosine. Three samples of piggery wastes were compared in this experiment (**A, B,** and **C**). The accumulation of these metabolites was not observed in the control slurries.

Table 1
Maximal Accumulation of Metabolites in Three Samples of Piggery Wastes

	Phenol	Para-cresol	Benzoate
A	4.6	1.6	6.2
B	3.7	1.26	0.43
C	5.0	2.3	4.8

[a]Five millimeters of L-Tyrosine were added in three samples of piggery wastes (A, B, and C). This table presents the maximal accumulations (mmol/L) of phenol, *para*-cresol, and benzoate that have been measured. These results represent the substraction of the concentration of the metabolites before their accumulation from their concentration before their degradation.

Figure 2A shows that 4HPPyrA and 4HPAA were nearly inactive as precursors of phenol in the first sample of slurry. Phenol was only significantly produced from L-tyrosine. The maximal concentration observed was 1.88 mmol/L after 17 d.

In another slurry (Fig. 2B), the phenol was produced from L-tyrosine, 4HPPyrA, and 4HPAA. A low accumulation of phenol was even observed in the control slurry (the concentration raised from 0.12–0.36 mmol/L at the 20th day). The maximal concentration of phenol was observed when

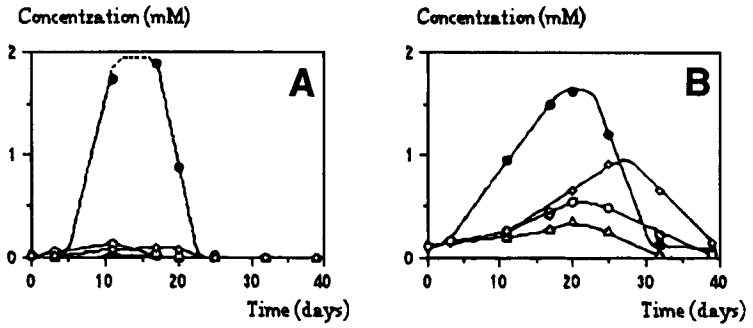

Fig. 2. This figure describes the accumulation of phenol in two samples (**A** and **B**) of piggery wastes. Three precursor metabolites were tested: L-tyrosine (●), 4HPPyrA (○), and 4HPAA (◇). A comparison is made with a control slurry (Δ).

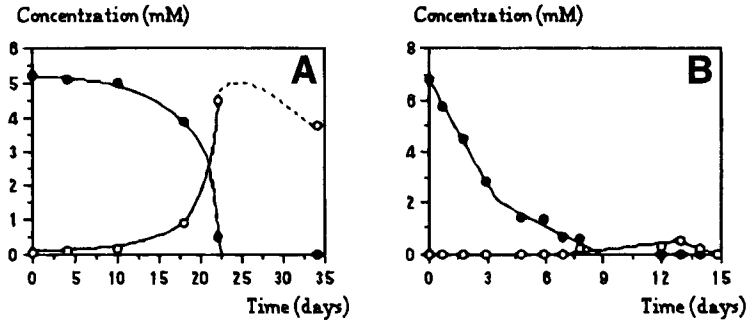

Fig. 3. Phenol was added in two samples (**A** and **B**) of piggery wastes at the approximative concentration of 5 m*M*. The evolution of phenol (●) and benzoate (○) concentrations was measured. 4HBA concentrations were measured in sample B only: no accumulations have been observed.

L-tyrosine was used as precursor: 1.63 mmol/L after 20 d. The degradation of 4HPPyrA and 4HPAA was observed with a delay compared to L-tyrosine. Maximal concentrations of phenol were 0.53 and 0.90 mmol/L after the addition of 4HPPyrA and 4HPAA, respectively.

Degradation of Phenol

The degradation of phenol was observed in several samples of piggery wastes. Figure 3 presents two different cases. In the case of an unadapted slurry (Fig. 3A), the degradation of phenol was slow in the first day and reached an average rate of 0.58 m*M*/d between days 18 and 22. In the same time, benzoate was produced at the average rate of 0.90 m*M*/d. It appears obvious that phenol is completely converted to benzoate. In the case of an adapted slurry (Fig. 3B), the degradation of phenol was achieved through two successive phases. During the first 3 d, the degradation average rate was about 1.36 m*M*/d. It decreased to 0.26 m*M*/d during the fol-

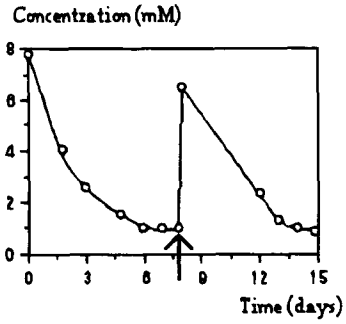

Fig. 4. The degradation of benzoate is observed in a sample of piggery waste. The arrow indicates a second feeding with approx 5 m*M* of benzoate.

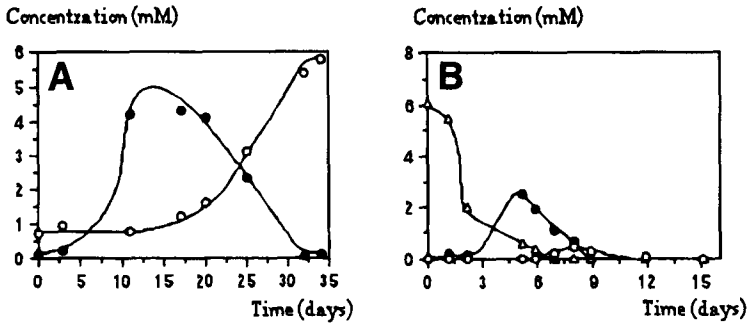

Fig. 5. This figure presents the fate of 4HBA (Δ) in two samples (**A** and **B**) of piggery wastes. The accumulation of phenol (●) and benzoate (○) was measured.

lowing days. The degradation was complete between the days 9 and 12. In contrast to case A, only weak amounts of benzoate were observed. The maximum was 0.51 m*M* after 13 d.

Degradation of Benzoate

The degradation of benzoate was also studied. The case presented in Fig. 4 was observed in the adapted slurry that had been used for Fig. 3B. The benzoate degradation began quickly (2.11 m*M*/d), before slowing and even ceasing between days 6 and 8. A second addition of benzoate—indicated by an arrow on Fig. 4—at day 8 restored the degradation activity.

Degradation of 4HBA

We observed the degradation of 5 m*M* of 4HBA in the adapted and unadapted slurries previously used for phenol and benzoate degradation experiments. The same slurry was used to obtain the results in Figs. 3B, and 5B. Figure 5A shows that 4HBA was completely converted to phenol and that phenol was consequently completely converted to benzoate. It was impossible to measure directly the disappearance of 4HBA with our

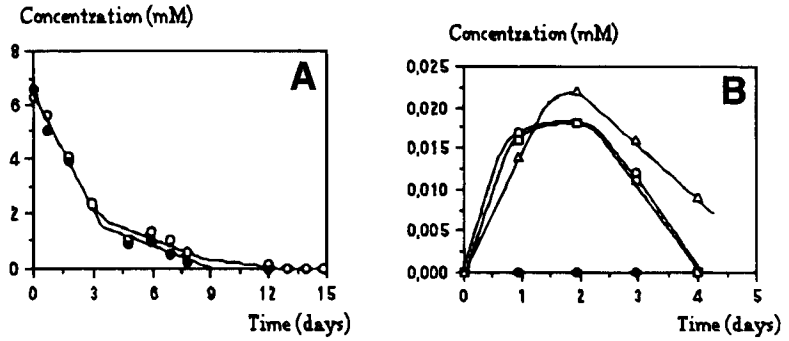

Fig. 6. A sample **(A)** of slurry was fed with both phenol and benzoate at the approximative concentration of 5 m*M*. Phenol (●) and benzoate (○) degradation were observed. In a second sample **(B)**, the addition of the metabolites was repeated, but benzoate was added at various concentration. B shows the accumulation of benzoate in each flask (5 m*M* ○, 10 m*M* □, or 20 m*M* of benzoate Δ). A comparison is made with a control slurry in which only phenol was added (●).

GC method. The accumulation of phenol reached the average rate of 0.50 m*M*/d. Between days 20 and 32, benzoate accumulation and phenol disappearance rates were almost the same: 0.30 and 0.34 m*M*/d.

Case B (Fig. 5B) presents the measurement of 4HBA uptake by HPLC. After a short lag phase, 4HBA concentration decreased at the average rate of 3.46 m*M*/d. The later diminution was lower: 0.42 m*M*/d between days 2 and 9. The phenol began to accumulate in the medium after 2 d. The maximal concentration observed was 2.5 m*M* after 5 d. After this moment, a diminution of 0.60 m*M* of phenol/d was observed. Phenol had completely disappeared after 10 d. Benzoate accumulated in weak amounts: the maximum observed was 0.46 m*M* after 8 d.

Simultaneous Degradation of Phenol and Benzoate

When phenol and benzoate were added simultaneously to the slurry, they were degraded at the same rate. This is shown in Fig. 6A. During the first 3 d, 1.46 m*M* of phenol and 1.33 m*M* of benzoate disappeared/d. During the later week, the disappearance rate was 0.43 m*M*/d for phenol and 0.37 m*M*/d for benzoate. No accumulation of 4HBA was observed in the same time.

In a similar experiment, phenol was added alone (control) or with various concentrations of benzoate (5, 10, or 20 m*M*). 4HBA could not be detected in the control flask. In the other flasks, 4HBA accumulated in weak concentrations. The maximum was observed in the flask where 20 m*M* of benzoate were added: 22 μmol/L after 2 d (Fig. 6B).

DISCUSSION

As demonstrated in Fig. 1, L-tyrosine is the precursor metabolite of phenol, benzoate, and *para*-cresol. These degradation products were observed in the three samples studied. This was confirmed in later studies

concerning more than 20 samples of piggery wastes from different piggeries (results not published). These reactions can then be considered as ubiquitous.

The degradation pathways of L-tyrosine to phenol were investigated. As indicated in Fig. 2A, a significant accumulation of phenol was observed after the addition of L-tyrosine, although it was not the case after the addition of 4HPPyrA or 4HPAA. This indicates that in this slurry, L-tyrosine is produced through a single reaction, probably by the mean of a tyrosine phenol lyase, as has been described for *Clostridium tetanomorphum (2)*.

However, an alternative pathway including 4HPPyrA and 4HPAA possibly exists. It could explain the traces of phenol observed in the case of Fig. 2A and its significant amounts in the case of Fig. 2B after the addition of those two possible intermediate metabolites.

The relationships between phenol and benzoate were investigated. The conversion of phenol to benzoate is described in Fig. 3. We have strong arguments to conclude that 4HBA is not an intermediate compound in the conversion of phenol to benzoate in the piggery waste samples we studied.

Phenol is converted to benzoate without any accumulation of 4HBA, and 4HBA was decarboxylated to phenol, but never directly dehydroxylated to benzoate. If we imagine a two-stages pathway in which 4HBA is the intermediate compound, the fact that 4HBA is not detected must be owing to its quick transformation to benzoate. It was then interesting to observe the effect of an addition of 4HBA in a phenol-adapted slurry. Under such conditions, it was expected that 4HBA would have been directly converted to benzoate. On the contrary, we observed an accumulation of phenol as shown in Fig. 5B. Furthermore, benzoate was only detected after 6 d. It seems impossible then to consider 4HBA as an intermediate metabolite.

In later experiments, slurry was fed simultaneously with 5 m*M* of phenol and 5 m*M* of benzoate. It was expected that the presence of benzoate could modify the equilibrium of the reaction and allow the accumulation of 4HBA. As shown in Fig. 6A, phenol and benzoate were curiously degraded at the same rate. This indicates a strict relationship in their uptake and metabolism. It is probable that these metabolites are degraded by the same microorganism.

The effect of an increase in phenol concentration was the accumulation of 4HBA in very low concentration (Fig. 6B). In this experiment, 4HBA was observed with 5 m*M* of benzoate, although it was not the case previously. We think that it is probably owing to a low reversibility of the reaction of decarboxylation of 4HBA to phenol. Indeed, phenol uptake decreased when 4HBA disappeared from the medium (results not shown). This indicates that 4HBA is transformed to phenol rather then to benzoate.

Zhang and Wiegel *(12)* proposed that 4HBA decarboxylation to phenol and phenol conversion to benzoate was realized by the same microorganism. This is another strong argument for the proposal that 4HBA is not

included in the phenol degradation pathway. In such a case, the dehydroxylation of 4HBA is the only way to degrade it.

However, the role of such a conversion is not clear, since 4HBA and phenol are not used as carbon sources. Figures 3A and the 5A effectively show that in unadapted slurries, 4HBA and phenol were completely converted, 5 m*M* of 4HBA leading to the accumulation of 5 m*M* of benzoate. Although the phenol may be seen as an electron acceptor, it is not true for 4HBA, as shown by the two reactions below:

$$HO\text{–}C_6H_4\text{–}COOH \longrightarrow C_6H_5\text{–}OH + CO_2 \quad (1)$$

$$C_6H_5\text{–}OH + CO_2 + 2H^+ + 2e^- \longrightarrow C_6H_5\text{–}COOH + H_2O \quad (2)$$

If the benefit from 4HBA conversion was only to obtain phenol as an electron acceptor, we could expect an immediate utilization of phenol. This is not the case. We think that 4HBA decarboxylation to phenol causes its own benefits to the cell.

The fact that these metabolites are not used as carbon sources is also an argument to assume that they are cosubstrates in a cometabolism. This was already previously proposed by Béchard et al. *(13)* and Bisaillon et al. *(14)*.

In conclusion, L-tyrosine is degraded to phenol in one stage, probably by a tyrosine phenol lyase activity. An alternative pathway possibly exists. It includes 4HPPyrA and 4HPAA.

The phenol is converted to benzoate in one stage. 4HBA is not an intermediate compound in this reaction. This metabolite is converted to phenol, and this is probably the final step to phenol and benzoate of an unidentified compound. Since these reactions were observed in all the slurries we have studied, they probably reflect a general phenomenon.

Our further investigations will concern the isolation of the microorganisms implied in the degradation pathways, and especially in the conversion of 4HBA to phenol. We will also investigate the possible conversion of 4HPAA to phenol.

ACKNOWLEDGMENT

This work was supported by IRSIA-IWONL.

REFERENCES

1. Schaefer, J. (1977), *Agriculture and Environ.* **3,** 121–127.
2. Brot, N., Smit, Z., and Weissbach, H. (1965), *Arch. Biochem. Biophys.* **112,** 1–6.
3. Elsden, S. R., Hilton, M. G., and Waller, J. M. (1976), *Arch. Microbiol.* **107,** 283–288.
4. Spoeltra S. F. (1978), *Appl. Environ. Microbiol.* **36, no. 5,** 631–638.
5. Spoeltra S. F. (1977), *J. Sci. Fd. Agric.* **28,** 415–423.
6. Godefroid, J., Antoine, P., Anselme, P., Van Rolleghem, P., De Poorter, M.-P., Van de Woestyne, M., Verstraete, W., and Thonart, P. (1992), *Med. Fac. Landbouww Univ. Gent.* **57/4a,** 1717–1720.
7. Balba, M. T., Clarke, N. A., and Evans W. C. (1979), *Biochem. Soc. Trans.* **7,** 1115–1116.

8. Neufeld, R. D., Mack J. D., and Strakey J. P. (1980), *J. WPCF* **52, no. 9,** 2367–2377.
9. Grbic-Galic, D. and Vogel, T. M. (1987), *Appl. Environ. Microbiol.* **53, no. 2,** 254–260.
10. Tschech, A. and Fuchs, G. (1987), *Arch. Microbiol.* **148,** 213–217.
11. Sharak-Genther, B. R., Townsend, G. T., and Chapman, P. J. (1989), *Biochem. Biophys. Res. Commun.* **162, no. 3,** 945–951.
12. Zhang, X. and Wiegel, J. (1990), *Appl. Environ. Microbiol.* **56, no. 4,** 1119–1127.
13. Béchard, G., Bisaillon, J.-G., Beaudet, R., and Sylvestre, M. (1990), *Can. J. Microbiol.* **36,** 573–578.
14. Bisaillon, J.-G., Lépine, F., and Beaudet, R. (1991), *Can. J. Microbiol.* **37,** 573–576.