MASTER of SCIENCE MICROBIOLOGY

ABSTRACT

L.D. Levac

FACTORS AFFECTING THE BIOLOGICAL DEGRADATION OF STYPHNIC ACID

A mixed culture of microorganisms metabolizing styphnic acid was isolated from soil. The disappearance of the substrate was followed by spectrophotometry and by thin layer chromatography. It was shown that styphnic acid did not accumulate inside the cells but was metabolized.

Several factors influencing the degradation of this substrate were studied. Degradation was shown to occur in a complex medium containing yeast extract or vitamin free casamino acids. The mixed culture degraded styphnic acid under microaerophilic conditions, at temperatures from 15 to 35 C. The pH range varied from 6.5 to 9.0 with best conditions around pH 7.5. Substrate concentrations ranging from 50 to 150 µg/ml of styphnic acid were found to be most satisfactory for efficient metabolization. Of several commercial nutrients tested, only corn steep liquor was found to replace adequately vitamin free casamino acids.

No intermediates were detected during the metabolization of styphnic acid. The mixed culture was tested for its ability to degrade a variety of nitrophenols. In a practical trial, a continuous culture system was tested and found to be more efficient than batch culture.

Short title: THE BIOLOGICAL DEGRADATION OF STYPHNIC ACID.

FACTORS AFFECTING THE BIOLOGICAL DEGRADATION OF STYPHNIC ACID

by

L.D. Levac

A THESIS

Submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfilment of the requirements for the degree of Master of Science.

Department of Microbiology,
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Québec.

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MASTER of SCIENCE MICROBIOLOGY

Résumé

L.D. Levac

ETUDE DES FACTEURS QUI INFLUENCENT LA DEGRADATION BIOLOGIQUE DE L'ACIDE STYPHNIQUE.

Une souche mixte de microorganismes capable de métaboliser l'acide styphnique fut isolée à partir de terres. La disparition de ce substrat fut suivie par spectrophotométrie et par chromatographie sur couche mince. On démontra qu'il n'y avait pas accumulation d'acide styphnique à l'intérieur des cellules mais plutôt une métabolisation.

Une étude des facteurs qui influencent la dégradation de ce substrat fut entreprise. La métabolisation n'a lieu que dans un milieu complexe qui contient un extrait de levure ou des acides casaminées sans vitamine. La souche mixte métabolise l'acide styphnique dans des conditions microaérophiles à des températures qui varient de 15 à 35C et à des pH situés entre 6.5 et 9.0. C'est à des concentrations de substrat entre 50 et 150 µg/ml que la métabolisation se révéla la plus efficace. Parmi les sources de nourritures commerciales qui furent utilisées dans l'espoir de remplacer les acides casaminées sans vicamine, seul un extrait de maïs s'avéra efficace.

Aucun produit intermédiaire ne fut détecté pendant la métabolisation du substrat. On vérifia aussi le pouvoir de métabolisation de cette souche mixte vis à vis différents nitrophénols. Comme solution practique on démontra les avantages d'une dégradation par culture continue.

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Special thanks are extended to my wife for her patience and understanding throughout this period. The manuscript was skilfully typed by Mrs. E. Rowell.

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INTRODUCTION

The persistence of synthetic chemicals in natural environments, particularly in soil and water, is a problem of considerable concern. Pesticides, detergents, packaging materials, and industrial wastes may reside in a particular ecosystem or move through a number of environments because of the inability of microorganisms to degrade the unnatural compound at significant rate, if at all. These chemicals are potential or actual pollutants of soil and water.

Nitro-substituted aromatic compounds may have diverse application like dinitro-o-cresol in agriculture or like styphnic acid in industry. Styphnic acid (2,4,6-trinitroresorcinol) is prepared industrially first by sulfonating, then nitrating resorcinol. It is used to identify organic compounds through their styphnate derivatives and as a primary explosive. The disposal of washings into rivers from reaction vessels after the industrial preparation of styphnic acid may cause a serious problem: beside being degraded at an insignificant rate, if at all, it may combine at trace concentrations with chlorine from water purification plants to give a bad taste to drinking water.

Chemical degradation of styphnic acid is practised by some industries using a catalytic reductive process; an expensive method. The biochemical oxygen demand (BOD) is lowered after this treatment, but no studies were apparently undertaken to test if the benzene

ring was cleaved or if the reduced compounds were less toxic than styphnic acid; the catalyst, for example, zinc may constitute a pollutant in itself.

The purpose of this investigation was to isolate by selective enrichment techniques microorganisms that degrade styphnic acid and to study the factors affecting this degradation.

LITERATURE REVIEW

Very little is known about the biological transformation of organic nitro-compounds. They are generally toxic and have a pronounced inhibitory effect upon the processes of assimilation in cellular metabolism. The effect of dinitrophenol on cell respiration is well known (Simon, 1953).

BIOSYNTHESIS

The antibiotic chloramphenicol, produced by *Streptomyces venezuelae*, is known to contain a NO_2 group attached to a benzene ring (Raistrick, 1949); this was probably the first known example of biosynthesis of an organic nitro-compound. Carter and McChesney (1949) identified β-nitropropionic acid as a hydrolysis product of certain glucosides from higher plants, and Bush *et al.*, (1951) showed that the same compound was synthesized by *Aspergillus flavus* in a corn steep liquor medium. Hirata *et al.*, (1954) reported that a toxic compound synthesized by *Streptomyces thioluteus* contained a nitro-benzene moiety similar to that of chloramphenicol.

Little is known about the biosynthesis of nitro-compounds. On the basis of experiments with mutant strains of *Neurospora crassa*, de La Haba (1950) suggested a pathway of nitrate and nitrite assimilation which involved the incorporation of inorganic nitrogen into an organic molecule before reduction to the amino level, presumably

as nitro groups. Westlake, *et al.*, (1968) showed, by using competitive sources of nitrogen, the absence of a preferred incorporation of ^{15}N from potassium nitrate-^{15}N into the nitro group of chloramphenicol suggesting that this substituent does not originate from a biological nitration reaction. Both nitrogen atoms of chloramphenicol appear to be derived from a common pool.

BIODEGRADATION

a) Utilization of nitro-compounds

Moore (1949) mentioned two unspecified proactinomycetes capable of utilizing nitrobenzene as simultaneous source of carbon and nitrogen.

Chambers and Kabler (1964) used phenol-adapted cultures of *Pseudomonas*, *Achromobacter*, *Xanthomonas* and *Flavobacterium* to study the degradation of various phenolic compounds. Bacterial suspensions of mixed cultures were adapted to and grown with a specific phenolic compound as the sole source of carbon and were tested by respirometric techniques to determine their ability to degrade related compounds. A positive test was one showing an uptake above 50 µl of oxygen over a 3-1/2 hour period. In general the nitrobenzoic acids were very resistant.

Alexander (1966) studied the effect of type, number or position of substituents on the rate of decomposition of the benzene ring by a mixed population. A mixed population of soil microorganisms was

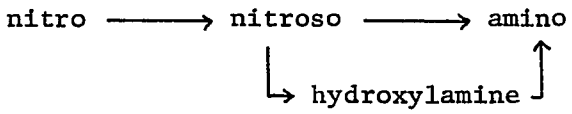
selected as the appropriate assay system to study the biological potential for the degradation of synthetic chemicals and to avoid the physiological limitations of individual microbial strains. The loss of ultraviolet absorbancy when the benzene ring was cleaved was used to assay for the degradation of the compounds. The test compounds were added at 5-15 µg/ml level as sole carbon source to a chemically defined medium. The time taken for the decomposition of nitro- and dinitrobenzene was over 64 days, except when the compounds contained a carboxyl or a phenolic hydroxyl group. Greater resistance was observed with the o- isomer of nitrophenol, less with p-, and least with the m- isomer. The author recognized some shortcomings of his experiments: the fact that no growth factors were used, that the pH, the temperature, and the concentrations of the substrates were not varied. Measurement of loss of UV absorbancy did not distinguish between degradation of the substrate and the use of the substrate for energy generation and synthesis of cellular material by the microorganisms.

Gundersen and Jensen (1956) isolated a strain of *Arthrobacter simplex* which degraded p-nitrophenol, 2,4-dinitrophenol, dinitro-o-cresol, and picric acid but was unable to attack a large variety of other nitro-compounds. He theorized that the breakdown of nitrophenols by this organism was conditioned on a para-orientation of the hydroxyl- and the nitro-groups on the benzene ring.

b) Detoxication of nitro-compounds

Enzyme systems in bacteria, fungi, and higher organisms can reduce several aromatic nitro-compounds to the corresponding arylamines. Also, a mammalian nitro reductase was reported by Fouts and Brodie (1957) in the highest concentration in liver, then in kidney followed by the lung and the heart. The enzyme was found in the microsome fraction and the soluble fraction of the homogenates. It transfers electrons from TPNH, less actively from DPNH to aromatic nitro-compounds such as chloramphenicol, nitrobenzoic acids, etc., and seems to be a special type of flavin enzyme.

The aerobic *Azotobacter*, the anaerobic *Clostridium butyricum*, and the fungus *Fusarium oxysporum* all reduce 2,4-dinitrophenol according to Radler (1955), Lehmberg (1956) and Madhosingh (1961) by the following pathway:

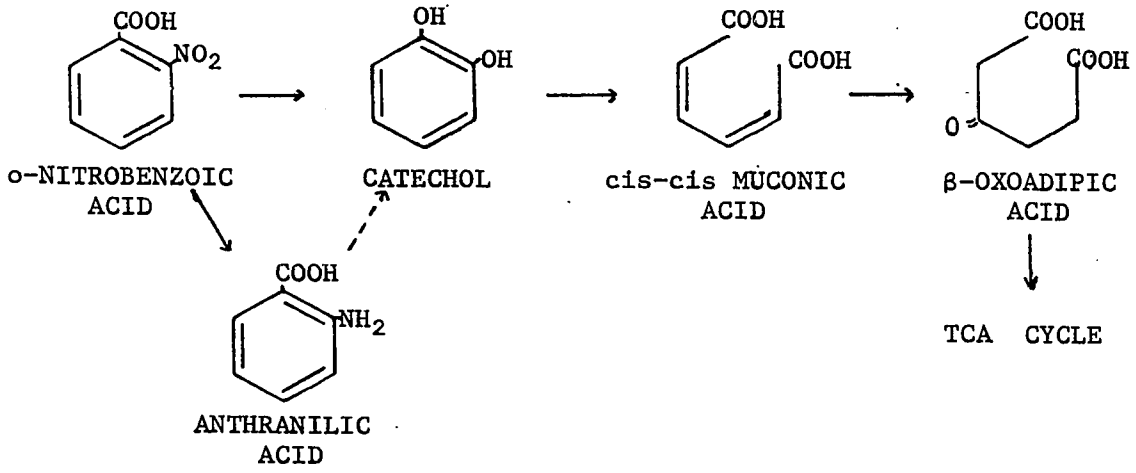


Villanueva (1961, 1964) found a *Nocardia* species capable of reducing several nitro-compounds and isolated and characterized the nitro reductase system.

Such processes may lower and even abolish the toxicity of nitro-compounds but represent only a superficial modification of the molecule and not a real decomposition to assimilable compounds.

c) Assimilation of nitro-compounds

Much less is known about the assimilation of aromatic nitro-compounds through their utilization as carbon and energy sources by microorganisms. The utilization of nitro- and amino-phenyl compounds as microbial carbon and nitrogen sources is known to occur in actinomycetes and pseudomonads (Simpson and Evans, 1953 ; Cain, 1958; Jensen and Lautrup-Larsen, 1967). In some cases reduction of the nitro group precedes assimilation (Durham, 1958); while in others the nitro group is released as nitrite ions (Gundersen and Jensen, 1956; Cain and Cartwright, 1960b). Cain (1966) showed that nitro-and amino-phenyl derivatives may be degraded in the early stages by quite independent metabolic routes, though these pathways converge at common hydroxylated intermediates.



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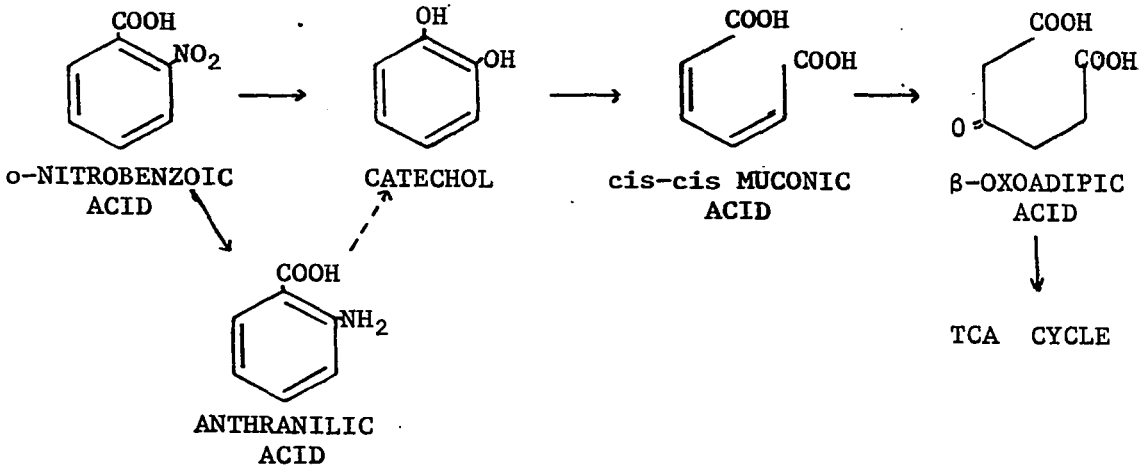


Anthranilic acid was shown not to be a direct intermediate in the metabolism of o-nitrobenzoate by *Nocardia opaca* although it appears transiently in the culture medium. Evidence that there is ring cleavage was demonstrated by the induction of catechol-1,2-oxygenases when the organisms were grown on o-nitrobenzoate. Thus even though nitro-aromatic compounds are very resistant structures, they can be utilized to generate energy by microorganisms.

d) Degradation of styphnic acid

Erickson (1941) showed that strains of *Micromonospora* isolated from lake mud were able to utilize an unspecified concentration of styphnic acid as carbon source as recorded by growth of the organisms. Chambers and Kabler (1964) reported that styphnic acid was not degraded as evidenced by a very low respiratory activity of their mixed culture. Franklin (1958) obtained biodegradation of 100 ppm of styphnic acid by an unidentified system isolated from soil over a one month period. Van Otterloo (1967) isolated several strains of microorganisms that would grow on media containing styphnic acid but the data reported do not show conclusively that styphnic acid was degraded and lyophilized cultures did not retain their ability to degrade the nitro-compounds. Cabridenc (1966) reported a 7% biodegradation of styphnic acid by a mixed culture of organisms over a 24 hour period.

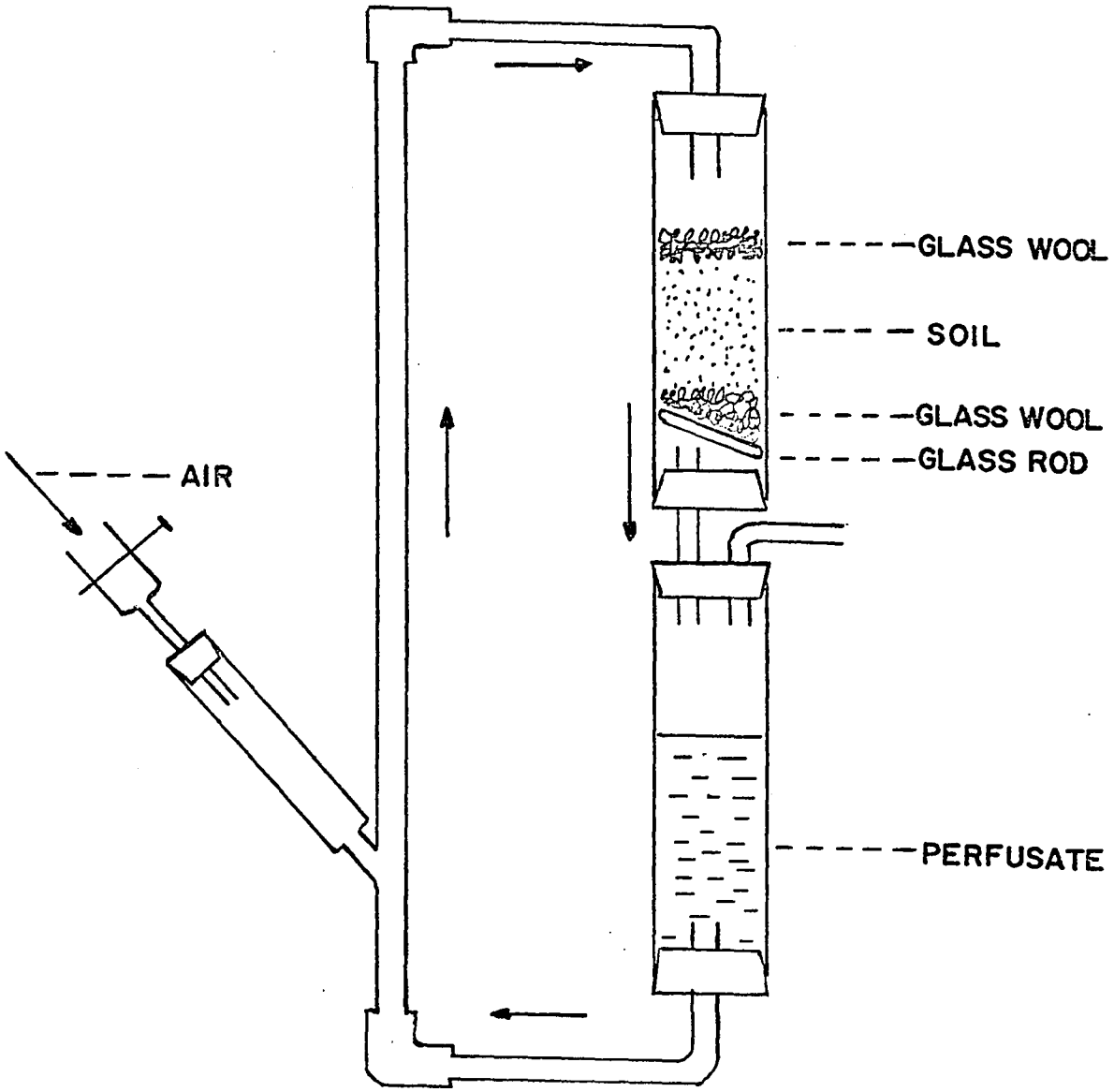
MATERIALS AND METHODS

ISOLATION OF THE ORGANISMS

The organisms used in this investigation were isolated with the perfusion method of Lees and Quastel (1944) from different soil samples. A series of soil samples were taken from corn fields of the Green Giant Company of Ste. Martine, P.Q., which had been sprayed with the herbicide dinitro-ortho-secondary butyl phenol. A second series of samples were taken from a sand field in l'Assomption, P.Q., which was utilized to burn phenolic residues from petroleum refineries. The last series of samples were collected from the Morgan Arboretum of Macdonald College. Thirty grams samples of wet soil were placed in a glass column. The soil columns were supported by glass-wool and perfused with the medium by forcing air through a sidearm tube and controlled by a screw clamp (Figure 1). A series of 12 columns were connected together with a glass manifold.

The disappearance of the substrate was checked at different time intervals by withdrawing samples from each perfusion apparatus, and after centrifugation and appropriate dilution, their absorption spectra were determined. The perfusate contained 0.15% K_2HPO_4, 0.1% of a trace elements solution (Umbreit, *et al.*, 1964), and 0.001% styphnic acid. The medium was adjusted to pH 7.0 and 180 ml of the perfusate was added to each percolator. During the analysis of the perfusate, a

Figure 1. Perfusion apparatus


GLASS WOOL
SOIL
GLASS WOOL
GLASS ROD
AIR
PERFUSATE

reference cuvette was set up which contained the inorganic medium.

When active metabolism was in progress as indicated by the disappearance of styphnic acid, subcultures were made into a medium containing the same salt composition as the perfusate, 0.005% styphnic acid and 0.5% yeast extract. The inoculated media were incubated at 25 C as stationary cultures. When growth was observed, a sample was serially diluted in saline and plated on nutrient agar plates and on the same medium used above for enrichment but with the addition of 1.5% agar. A plate was selected which contained about 40 colonies. All the colonies were picked up and subcultured on slants of the above media.

MAINTENANCE OF CULTURES

The mixed culture was maintained in a liquid medium by transfering periodically about 5 ml of the culture to 100 ml of fresh medium in a 250 ml Erlenmeyer flask and incubating it at 25 C. The liquid medium consisted of 0.005% styphnic acid, 0.15% K_2HPO_4, 0.5% vitamin free casamino acid (Difco Laboratories), and 0.1% trace elements solution.

The pure isolates were subcultured on slants of the above medium to which was added 1.5% agar.

PREPARATION OF THE INOCULUM

A 250 ml Erlenmeyer flask containing 100 ml of the above liquid medium was inoculated with 5 ml of a suspension of the mixed culture whose supernatant showed 0.1 optical density units at 412 mμ. The cells were grown for 48 hours at 25 C. Five millilitres of this growing culture were transferred into 500 ml Erlenmeyer flasks containing 300 ml of the liquid medium. The cells were again allowed to grow for 48 hours at 25 C after which they were centrifuged aseptically for 20 minutes at 10,400 x g on a Sorvall RC 2 centrifuge and washed three times with saline. The cell density was adjusted to 5% transmission on a Coleman Junior Colorimeter. Five millilitres of this suspension were used as the inoculum.

MEDIA

1. Standard medium

The standard medium consisted of styphnic acid added from a concentrated stock solution at the desired concentration, 0.15% K_2HPO_4, 0.1% of a trace element solution (Umbreit, *et al.*, 1964), 0.5% of vitamin free casamino acids in distilled water as supplemental carbon and nitrogen source.

2. Medium with synthetic amino acid mixture

An amino acid mixture approximating the amino acid composition

of casein (National Research Council, 1953) was prepared with L-form amino acids obtained from commercial sources. This mixture, equivalent to a 10% solution of casein, was added to the standard medium in order to replace vitamin free casamino acids.

3. Medium with sulfite liquor

Sulfite liquor was obtained from the Pulp and Paper Research Institute in Pointe Claire. The liquor was obtained from a pilot plant experiment and the sulfur analysis was as follows:

Total SO_2	5.7%
Free SO_2	4.6%
Combined SO_2	1.2%
pH	1.8

The liquor was neutralized with 40% NaOH (some precipitation occurred) and was used to replace vitamin free casamino acid in the standard medium.

4. Medium with malt extract

Non-diastatic malt syrup was obtained from Lallemand Ltée. in Montreal. The syrup was adjusted to 20 balling units with a hydrometer and was used to replace vitamin free casamino acids in the standard medium.

5. Medium with corn steep liquor

Corn steep liquor was supplied by the Ste. Lawrence Starch Company Limited of Port Credit, Ontario. The liquor was neutralized with 40%

NaOH, centrifuged for 20 minutes at 10,400 x g in a Lourdes centrifuge to remove the solid particles. The supernatant was filtered on a Whatman No. 1 filter before being finally filter sterilized with a Millipore filtering apparatus and THROUGH 0.45 μ filters. The corn steep liquor was used to replace the vitamin free casamino acids in the standard medium.

FERMENTATION CONDITIONS

Unless otherwise stated, all experiments were carried out with stationary cultures using 300 ml of the medium in 500 ml Erlenmeyer flasks. At time intervals, aliquots were removed, centrifuged, and assayed.

A continuous culture system (Figure 2) was devised as a practical application of the degradation of styphnic acid. It consisted of a 1 l. Erlenmeyer FLASK reservoir containing a concentrated solution of styphnic acid and nutrients connected to a glass column acting as a constant pressure system. A valve controlled the flow of concentrated medium from the column into 250 ml fermentation vessels equipped with a sidearm tube to take samples. The liquor from one fermentation vessel was allowed to overflow into another similar vessel and finally into a large sedimentation tank. Mixing of the medium was achieved with magnetic stirrers and a small magnet in the fermentation vessels. The apparatus was assembled in a constant temperature incubator.

Figure 2. Continuous culture apparatus

A Reservoir of concentrated nutrients

B Column providing a constant pressure

C Valve to regulate flow rate

D Sidearm tube for sampling

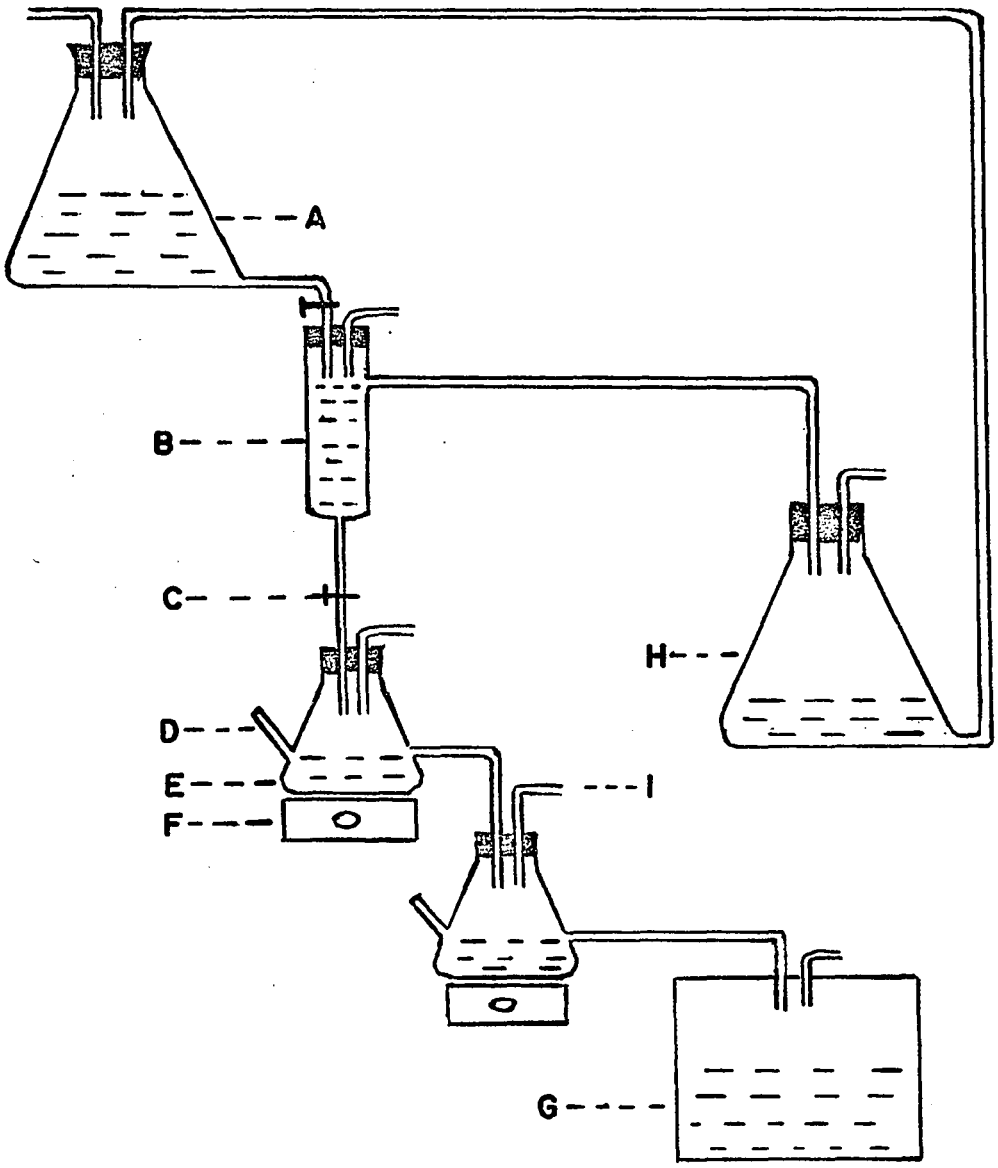
E Fermentation vessel

F Magnetic stirrer

G Sedimentation tank

H Overflow reservoir

I Tube to equilibrate pressure


A
B
C
D
E
F
G
H
I

ASSAYS

1. Growth

After initial centrifugation of the sample, the cells were washed three times with saline and resuspended to the original volume in saline. The absorption spectrum of the solution at 660 mμ was determined on a Coleman Junior Colorimeter in optically matched tubes after proper dilution to ensure that the reading would fall between 0.0 and 0.6 optical density units.

Dry weight determinations were done by washing and resuspending the cells in distilled water. Aliquots of varying volume were added to pre-weighed beakers and dried in an oven at 85 C. The dried samples were weighed on an analytical balance to constant weight. A standard curve was constructed relating dry weight to optical density with the same cell suspension.

2. Determination of pH

The pH of the samples was determined on a Beckman Zeromatic pH meter with a combination electrode.

3. Measurement of ammonia

Nessler's reagent (Cowan and Steel, 1965) was prepared by dissolving 8 g of potassium iodide and 11.5 g of mercuric iodide in 50 ml of ammonia free water. Fifty ml of 6 N NaOH was added: the solution was mixed and allowed to stand for 24 hours. Ammonia production was measured by dipping a piece of filter paper in the reagent and testing

for the presence of ammonia vapours in a flask containing cells growing on styphnic acid. A control consisted of a flask containing distilled water.

4. Measurement of nitrite

A modified Griess-Ilosvay method (Black, 1965) using sulfanilamide as the diazotizing agent and N-(1-naphthyl)-ethylene diamine hydrochloride as the coupling reagent was used to measure nitrite from fermentation samples.

5. Styphnic acid

Fermentation samples were analyzed qualitatively by thin layer chromatography after the extraction of styphnic acid with ethyl ether while quantitative determination of styphnic acid was done directly on the supernatant or after an ether extraction of the sample.

The extraction was carried as follows: one volume of the fermentation sample, after centrifugation, was extracted 3 times in a separatory funnel with one volume of ethyl ether after acidification of the sample below pH 2.0. The ether fraction was collected in a small beaker and the ether was evaporated using a stream of cold air from a commercial hair-dryer. The water fraction was transferred to a small beaker and was dried in a vacuum dessicator over phosphorus pentoxide. The efficiency of the extraction was found to be 100%.

a) Qualitative determination

Thin layer chromatography was used to follow the disappearance of styphnic acid from the medium and to detect the presence of intermediates. Glass plates of 20 x 20 cm were acid washed in a sulfochromic acid bath for 24 hours, rinsed with distilled water and dried with methanol. The slurry was prepared by mixing 15 g of micro-crystalline cellulose (Sigma Chemical Co.) in a Waring blender for 90 seconds. Five plates of 200 μ thickness were spread using a Quickfit apparatus. The plates were air-dried overnight and then heated at 80 C for 30 minutes. The plates were not stored in a dessicating cabinet since micro-crystalline cellulose does not readily absorb moisture. Pre-coated plates of micro-crystalline cellulose of 200 μ thickness were also purchased from Camag, Chemie-Erzeugnisse und Adsorptionstechnik AG.

The solvent used consisted of a mixture of 2 parts of absolute alcohol, 1 part of benzene, 1 part of 1-pentanol, and 1 part concentrated ammonium hydroxide (Madhosingh, 1961).

The dried samples obtained from the ether extraction procedure were redissolved in an equal amount of absolute alcohol. Equal aliquots of the fermentation samples were spotted on the plates using a 25 μl micropipette. The spots were dried using a cold stream of air from a commercial hair-dryer.

The plates were developed for 1-1/2 hrs by ascending chromato-

graphy in a rectangular chromatography tank saturated with the solvent. A piece of chromatography paper was layered around the inside of the tank to ensure uniform distribution of the vapour phase, thus preventing trailing about the edges. After separation of the compounds, the plates were air-dried in a fumehood.

Styphnic acid spots were readily detected and their intensity could be estimated by visual observation after the development of the plates since they produced yellow spots under alkaline conditions. The plates were then scanned under a Mineralight Ultraviolet lamp to detect any fluorescent spot. If required, the plates were sprayed with a 0.1% ethanolic solution of ninhydrin or with vanillin: toluene: p-sulphonic acid (VTPSA) (Roux and Maihs, 1960) which gives a pink to deep brown colour with phenols.

Chromatogram standards were prepared by dissolving 0.005 g of styphnic acid in 100 ml of absolute alcohol. Standards containing 25 µl were spotted as controls.

Thin layer chromatography detected less than one µg of styphnic acid.

b) Quantitative determination

Standard curve for styphnic acid

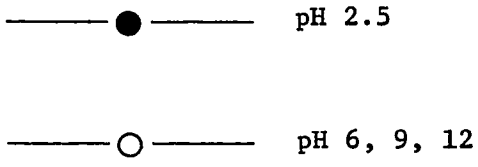
The absorption spectrum of a 0.005% solution of styphnic acid, diluted 1 : 5, was determined at various pH on a Unicam SP 800 Spectrophotometer in a 3 ml quartz cuvette of 1 cm path using

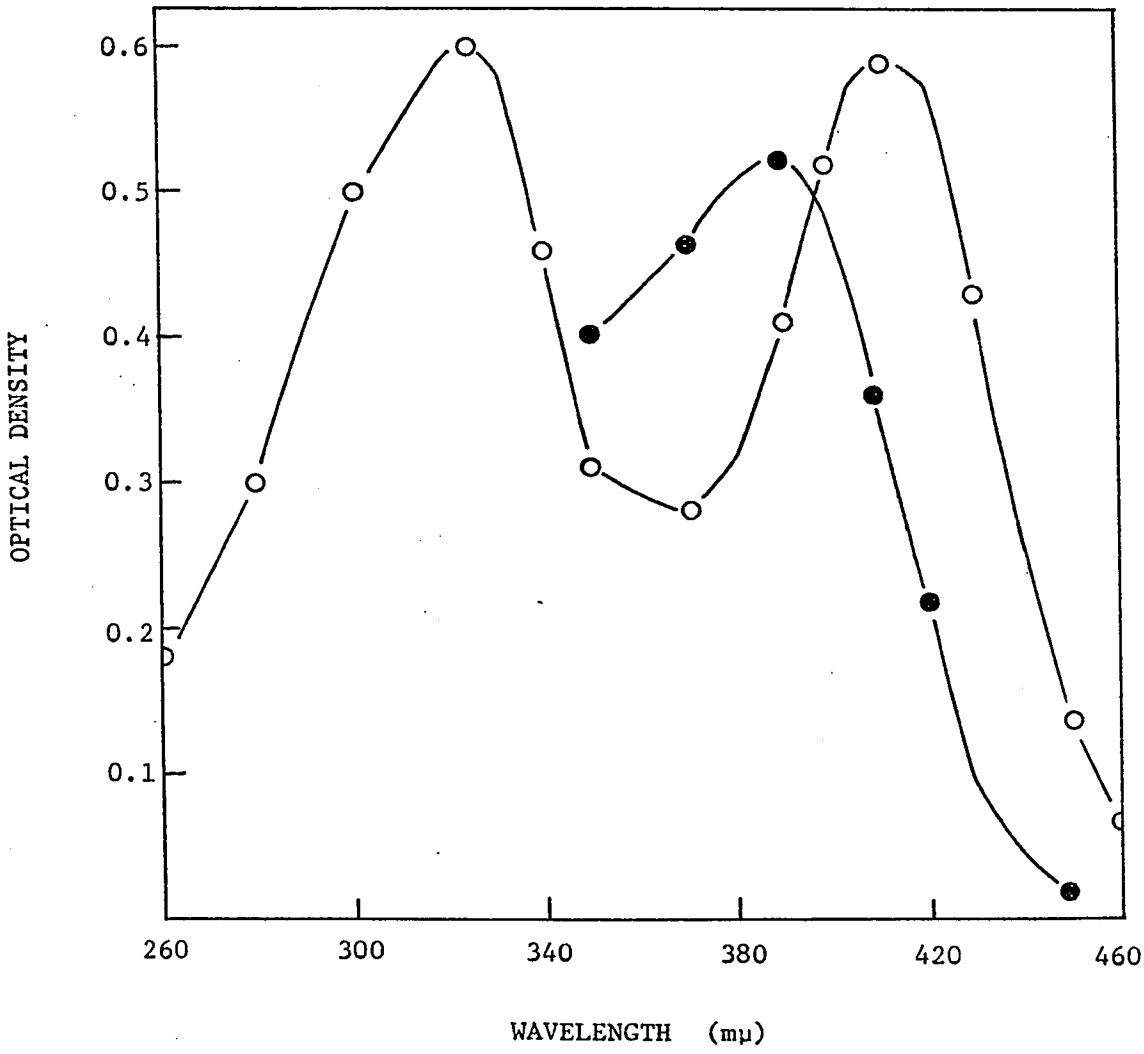
distilled water in the reference cuvette. The spectra of these solutions are shown in Figure 3. The absorption spectra of the solutions from pH 6.0 to pH 12 are identical while the spectrum at pH 2.5 is shifted to the left. Above pH 6.0 the peaks are stable and maximum colour intensity of the solutions is obtained. Two peaks were detected, one around 420 mμ and one around 325 mμ. The peak at 412 mμ under alkaline conditions, was selected for quantitative analysis because of its narrowness and its symmetry. A standard curve was constructed relating the optical density to different concentrations of styphnic acid. The absorption spectrum of the different concentrations was determined at 412 mμ on a Gilford 300 N Spectrophotometer over a range of 0 to 100 μg/ml. Figure 4 shows that there is a direct relationship between the optical density and the concentration of the solutions from 0 to 14 μg/ml of styphnic acid.

c) Preparation of samples

After centrifugation, the supernatant was diluted in order to obtain a reading within the range of the standard curve. The solution was made alkaline by adding NaOH so that the final concentration was 0.1 N NaOH. Styphnic acid concentration was determined at 412 mμ on a Gilford 300 N Spectrophotometer using 0.1 N NaOH in distilled water as a blank. When required, styphnic acid concentration was determined from the ether fraction after an

Figure 3. Absorption spectra of a styphnic acid solution at various pH on a Unicam SP 800 Spectrophotometer.




OPTICAL DENSITY
0.6
0.5
0.4
0.3
0.2
0.1
260
300
340
380
420
460
WAVELENGTH (mμ)

ether extraction as described.

6. Analyses of various nitrophenols

Qualitative and quantitative determinations were carried out for various nitrophenols. The procedures utilized were similar to those described before for the analysis of styphnic acid. Spectrophotometric measurements were determined at 412 mμ except for 2,4-dinitrosoresorcinol which was measured at 335 mμ. Figure 5 shows that there is a linear relationship from 0 - 30 μg/ml concentration for o- and m-nitrophenol, 2-nitroresorcinol, and picric acid; from 0 - 25 μg/ml concentration for 2,4-dinitrophenol, and from 0 - 10 μg/ml concentration for p-nitrophenol, and for 2,4-dinitrosoresorcinol.

HYDROLYSIS OF THE CELLS AND OF THE SUPERNATANT

A standard medium was seeded with the inoculum. Aliquots were removed at time intervals and divided into three equal portions which were centrifuged at 10,400 x g for 20 minutes in a Lourdes centrifuge. The supernatant of the first portion was analyzed directly for its styphnic acid content. The supernatant of the second portion, together with the redissolved sediment which was washed twice with saline, was used for acid hydrolysis. The third portion was treated as the second portion but was used for the alkaline hydrolysis.

Figure 4. Standard curve for styphnic acid measured at 412 mμ on a Gilford 300 N Spectrophotometer.

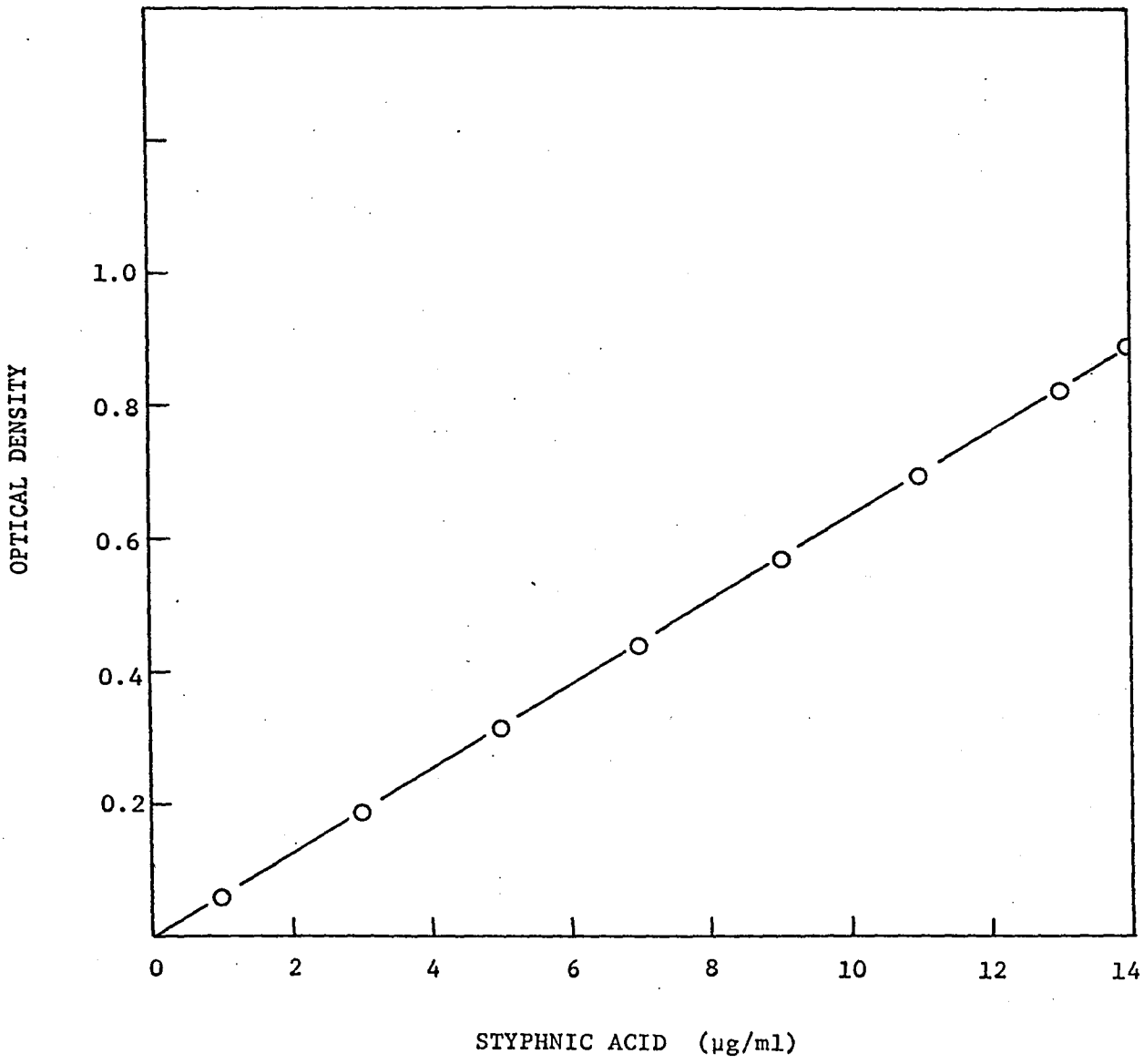
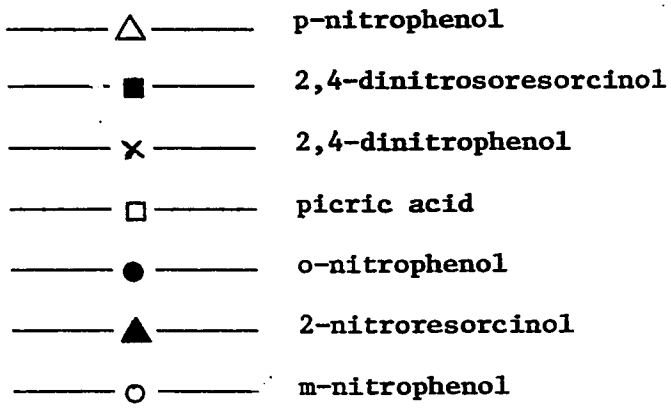
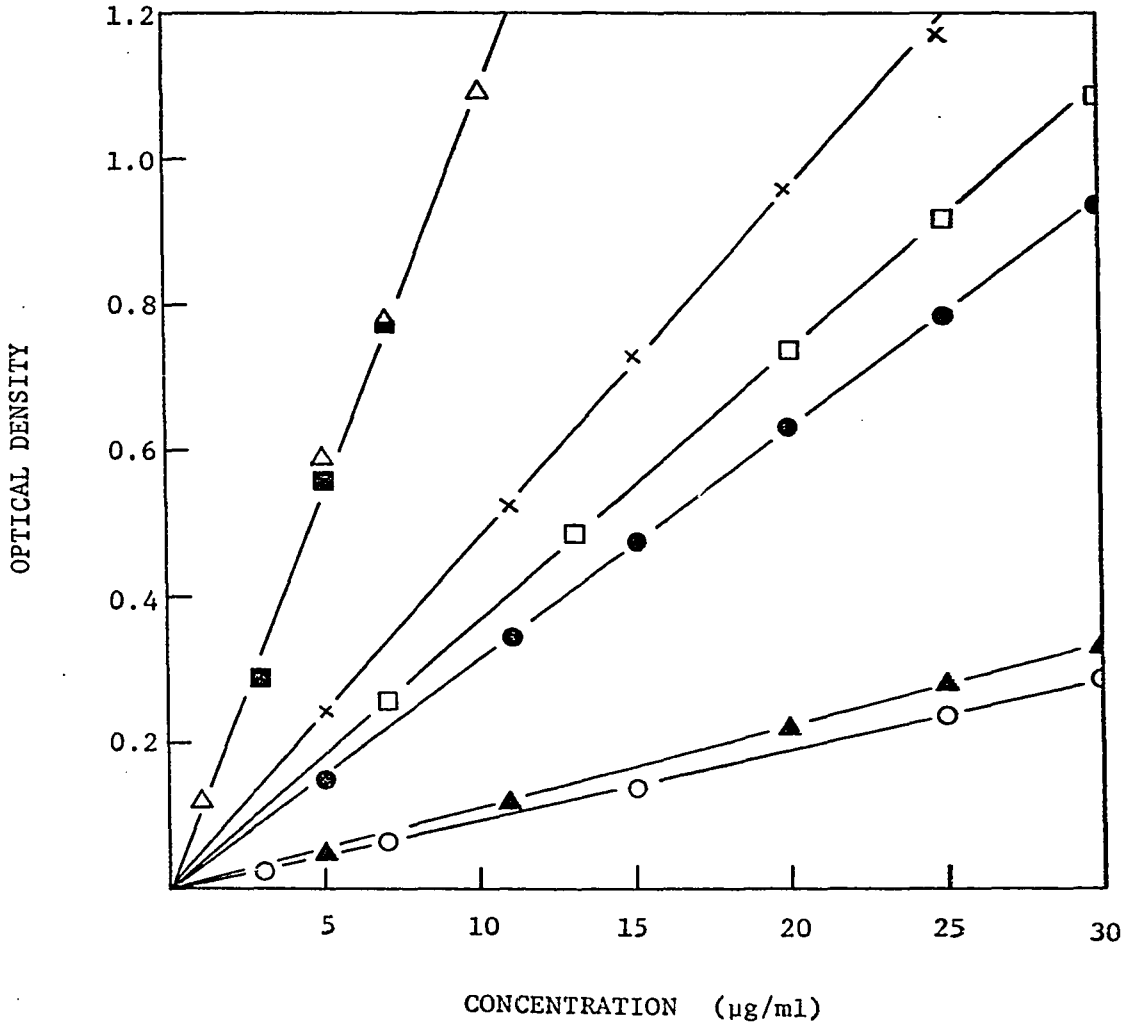

OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
14
STYPHNIC ACID (µg/ml)

Figure 5. Standard curve of different nitro-phenolic compounds measured at 412 mμ on a Gilford 300 N Spectrophotometer. 2,4-dinitrosoresorcinol was measured at 335 mμ.




1.2
1.0
0.8
0.6
0.4
0.2
OPTICAL DENSITY
5
10
15
20
25
30
CONCENTRATION (µg/ml)

1. Acid hydrolysis

Five millilitres of 6 N HCl were added to 10 ml of the supernatant and to 10 ml of the sediment in hydrolysis tubes (20-25 ml test tubes with a glass stopper). The tubes were held tightly between two test tube racks with wires. Hydrolysis was carried for 12 hours in an autoclave at 121 C and 15 psi. A control consisted of a 10 ml aliquot of the uninoculated growth medium treated similarly.

2. Alkaline hydrolysis

Five millilitres of 8 N NH_4OH were added to 10 ml of the supernatant and to 10 ml of the sediment in a 50 ml Claisen flask equipped with a reflux condenser. Heating under gentle boiling conditions was performed using a sand bath and a hot plate for 8 hours in a fumehood.

3. Post hydrolysis treatment

After hydrolysis the samples were brought to neutrality and diluted in volumetric flasks to equal volumes. The samples were examined by spectrophotometric analysis for styphnic acid, and after ether extraction by thin layer chromatography.

RESULTS

IDENTIFICATION OF MICROORGANISMS ISOLATED BY AN ENRICHMENT TECHNIQUE

Microorganisms degrading styphnic acid were isolated from various soils by an enrichment technique. The disappearance of styphnic acid was observed in percolator No. 8 as shown in Table 1. A soil sample was then enriched in standard medium with yeast extract. Upon microscopic examination of the enriched mixture degrading styphnic acid, spores were observed. Samples were heat treated at 80 C for 5, 10, and 15 minutes, and then serially diluted. The dilutions were plated on nutrient agar plates. After incubation at 25 C, a plate containing about 40 colonies was selected to provide pure cultures to be tested for their ability to degrade styphnic acid. After the transfer of each individual colony onto nutrient agar slants, the same plate was washed with saline and the resulting suspension was added to fresh standard medium. Styphnic acid degradation occurred with this mixed culture indicating that the organisms responsible for the disappearance of styphnic acid in the enrichment were recovered. The heat resistance of the bacteria indicated the necessity of identification of heat resistant microorganisms and the tests were performed according to Cowan and Steel (1965). The individual colonies were characterized. The results are presented in Table 2. Two of the six different organisms characterized were tentatively identified as members of the genus *Bacillus*. The four

TABLE 1. The disappearance of styphnic acid from soil percolators.

Percolator No.	Location of the soil sample	Pre-treatment of the soil sample	Styphnic acid (µg/ml)		
			0 day	7 days	16 days
1	Ste. Martine	Sineb* (0.75 gallons/acre)	10	10	10
2	Ste. Martine	Sineb (0.75 gallons/acre)	10	10	10
3	Ste. Martine	Sineb (0.75 gallons/acre)	10	10	10
4	Ste. Martine	Sineb (1.25 gallons/acre)	10	10	10
5	Ste. Martine	Sineb (2.5 gallons/acre)	10	10	10
6	L'Assomption	Phenolic residues	10	10	10
7	L'Assomption	Phenolic residues	10	10	10
8	Morgan Arboretum	- - -	10	7	3
9	Morgan Arboretum	- - -	10	10	10
10	Morgan Arboretum	- - -	10	10	10
11	Macdonald Farm	- - -	10	10	10
12	Macdonald Farm	- - -	10	10	10

* Sineb is the commercial name for dinitro-ortho-secondary butyl phenol.

TABLE 2. Characterization of the different isolates: Cultural characteristics.

	Culture No. 1	2	3	4	5	6
Nutrient broth	Pellicle Heavy sediment	Pellicle Heavy sediment	Pellicle Turbid growth Heavy sediment	Pellicle Turbid growth Small sediment	No pellicle Scant growth Small sediment	No pellicle Slight growth Small sediment
Nutrient agar plate	Raised colony 3-4 mm in diameter, rough surface, transparent edges with opaque center	Raised colony 2-3 mm in diameter, smooth surface, transparent edges, opaque center	Flat colony 3-4 mm in diameter, crispy opaque	Raised colony 2-3 mm in diameter, opaque white, shiny, creamy	Flat colony 3-4 mm in diameter, transparent edges with creamy center	Raised colony Pinpoint Soft
Standard medium slant	Scant growth	Scant growth	Scant growth	Scant growth	Scant growth	Scant growth
Standard medium plate	Same as on nutrient agar plates but the colonies are smaller.					
MORPHOLOGY						
Gram stain	+ve rod 1 x 4 µ	+ve rod 1 x 6 µ	Variable Short rod	+ve Short rod	Variable Rods from short to long	Variable Short rod
Spore stain	Central oval non-swelling spore	Central to sub-terminal non-swelling spore	-	-	-	-

(continued)

TABLE 2. (continued)

	Culture No. 1	2	3	4	5	6
MORPHOLOGY (cont'd.)						
Fat stain	-	-	-	-	-	-
Motility	+	+	+	-	+	+
FERMENTATION						
Arabinose	*AG	-	A	A	A	-
Glucose	**A	-	A	A	A	-
Mannitol	A	-	A	AG	A	-
OTHERS						
Indole	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Nitrate	+	+	+	+	+	-
V-P	-	-	-	+	-	-
Citrate	+	+	+	+	+	-
Gelatin	+	+				
Casein	+	+				
Urease	-	-				
Starch	+	-				

**A = acid

*AG = acid-gas

other different isolates were not identified. The mixed culture initially isolated was lyophilized and labelled as Mac No. 551.

STUDIES ON THE DEGRADATION OF STYPHNIC ACID BY ISOLATED CULTURES

Degradation of styphnic acid by pure cultures

One hundred millilitres of standard medium containing 50 µg/ml concentration of styphnic acid was added to 250 ml Erlenmeyer flasks. Each flask was seeded with an individual isolate and incubated at 25 C. None of the isolates was able to degrade styphnic acid even though growth of the organisms occurred. The addition of 0.1% yeast extract did not bring about any further degradation.

Degradation of styphnic acid by a combination of microorganisms

Suspensions of equal concentration of each isolate were prepared from organisms grown in the conditions stated above and all possible combinations of organisms were mixed together. For example let us consider that four isolates were used. The following combinations were then possible beside the use of individual isolates: 1+2, 1+3, 1+4, 2+3, 2+4, 3+4, 1+2+3, 1+2+4, 1+3+4, 2+3+4, 1+2+3+4. Equal amounts of the suspensions were added to the same medium in which they had been grown. Growth of the cells occurred in all trials but there was no degradation of styphnic acid. Partial degradation of styphnic acid (medium became slightly orange and a 5 - 10% degradation was inconsistently obtained) was obtained when all the isolates were added together.

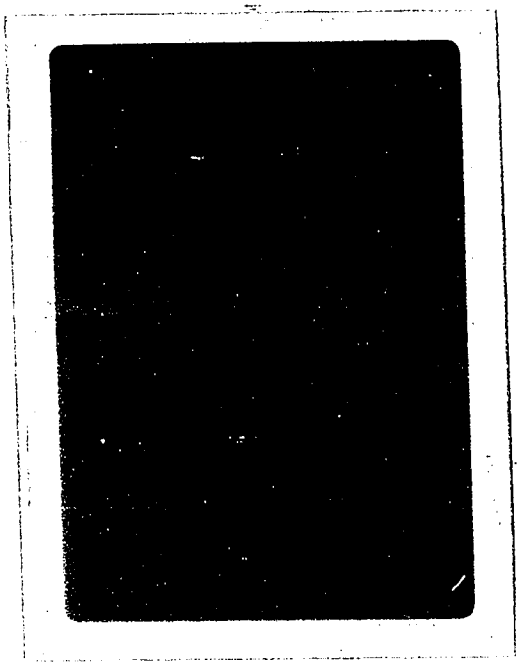
Degradation of styphnic acid by a mixed culture

A mixed culture which was originally isolated and subcultured from soil percolators was added to standard medium containing 44 µg/ml of styphnic acid and 0.5% yeast extract instead of vitamin free casamino acids and incubated at 25 C. Table 3 shows from spectrophotometric evidence that styphnic acid disappeared almost completely after 72 hours. The degradation could be followed visually because the fermentation undergoes a series of characteristic colours as illustrated in Figure 6. After inoculation the medium is yellow and slightly turbid. This is followed after about 24 hours by an increased turbidity and by an orange colour. After 48 hours the medium is reddish brown. Finally the medium is brownish grey at the completion of the degradation. Thin layer chromatography substantiated spectrophotometric evidence as shown in Figure 7. The substrate disappeared with increasing fermentation time as evidenced by the fading of the yellow spots having an identical Rf value as the styphnic acid control. After 72 hours, no substrate could be detected. The substrate was found only in the ether fraction and no styphnic acid was detected in the water fraction. Figure 8 shows the relationship between the growth of the cells and the disappearance of styphnic acid. There is no lag phase since the inoculum was already growing on styphnic acid. The degradation of the substrate occurred during exponential growth of the cells and is

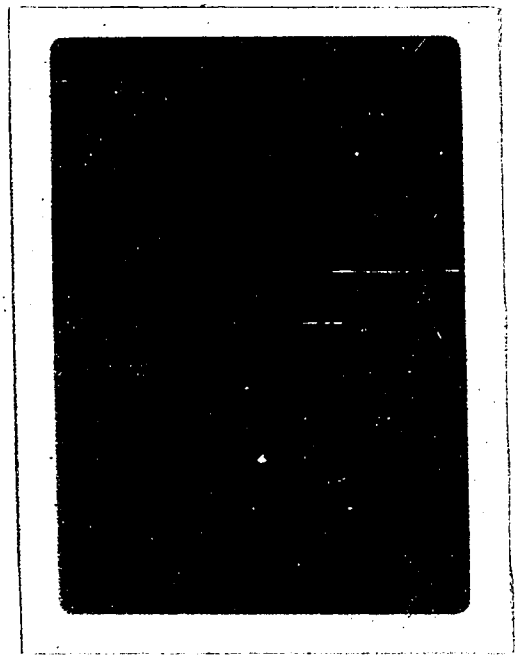
TABLE 3. Degradation of styphnic acid by a mixed culture of microorganisms at 25 C.

Time hours	Concentration of styphnic acid µg/ml
0	44
12	42
24	38
36	32
48	15
72	5

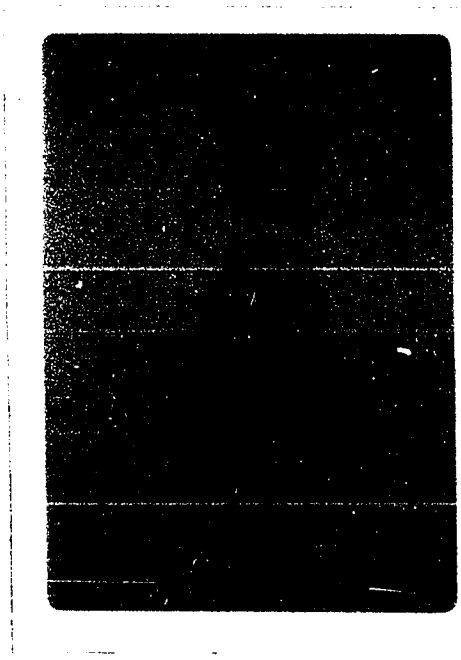
Figure 6. Typical colour changes during the degradation of styphnic acid by a mixed culture of microorganisms.



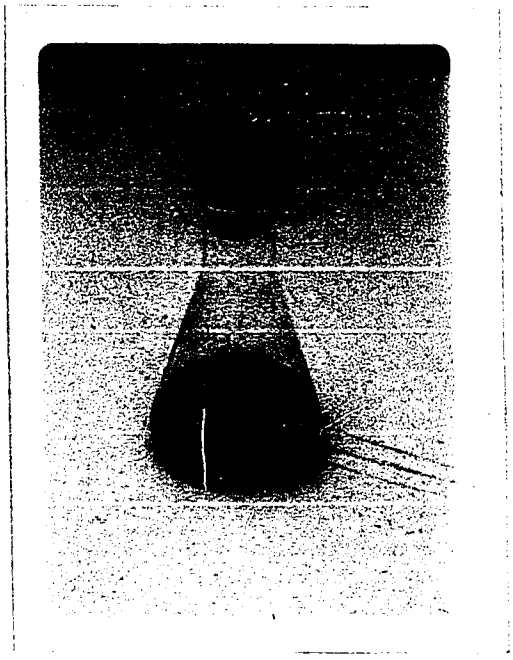
0 Hour



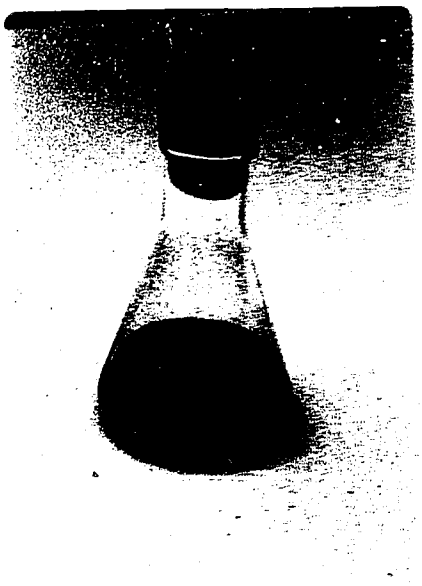
24 Hours



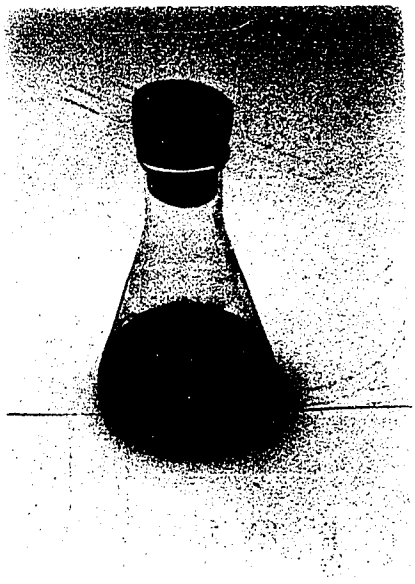
48 Hours



72 Hours

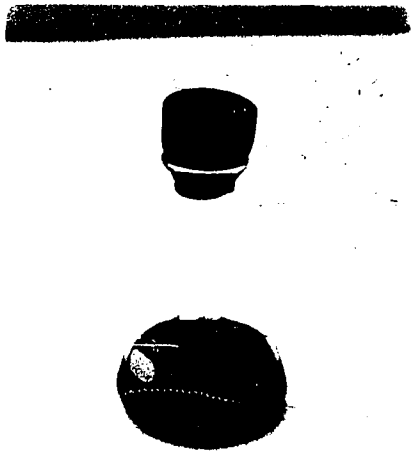


0 Hour



24 Hours

48 Hours



72 Hours

Figure 7. Chromatogram showing the disappearance of styphnic acid from the fermentation medium at 25 C.

1-0, 1-24, and 1-72 are the ether fractions of the 0, 24, and 72 hours samples.

2-0, 2-24, and 2-72 are the water fractions of the 0, 24, and 72 hours samples.

Spot no.	Colour	Colour intensity	Rf value	Identity
1	Yellow	++++	0.25	Control styphnic acid
2	"	++++	0.25	Sample spot
3	"	++	0.25	Sample spot

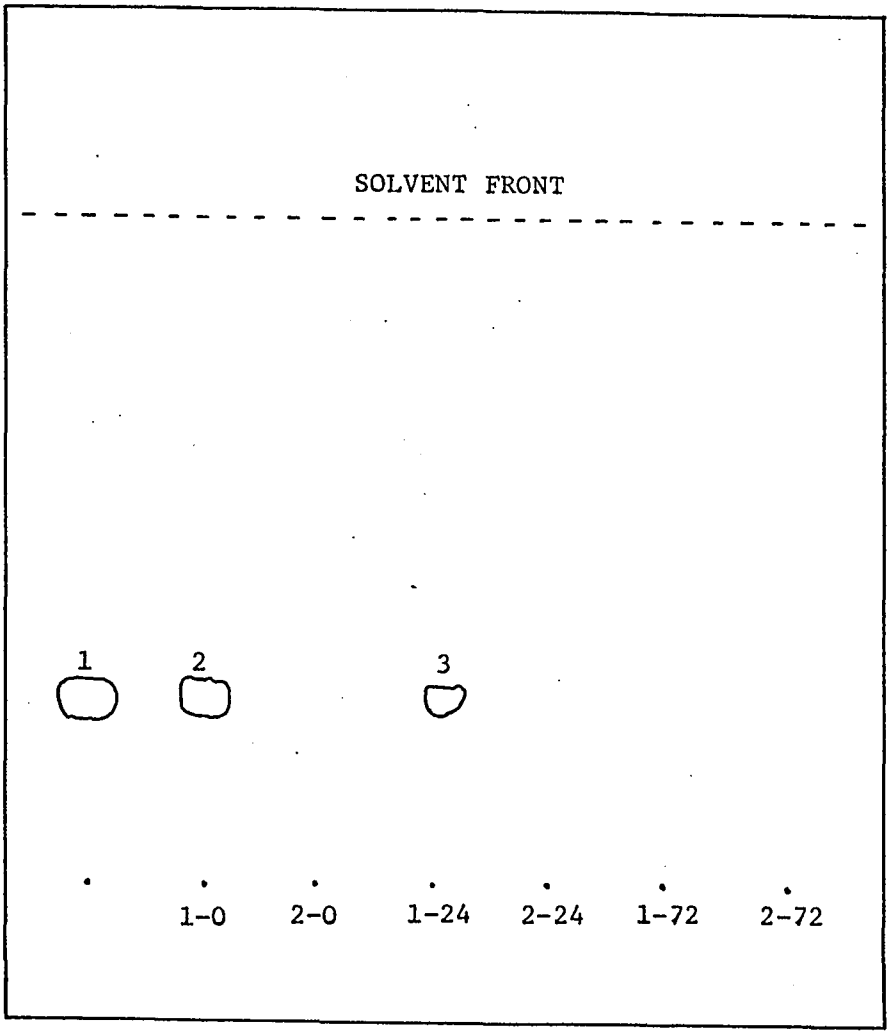
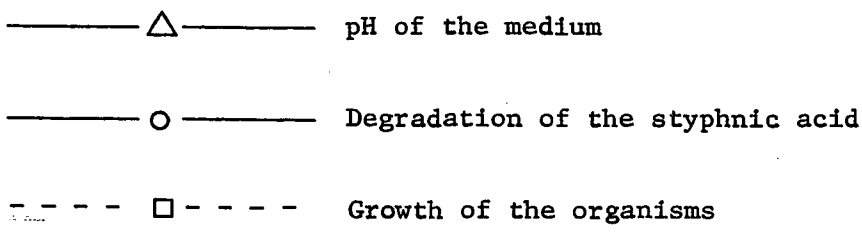
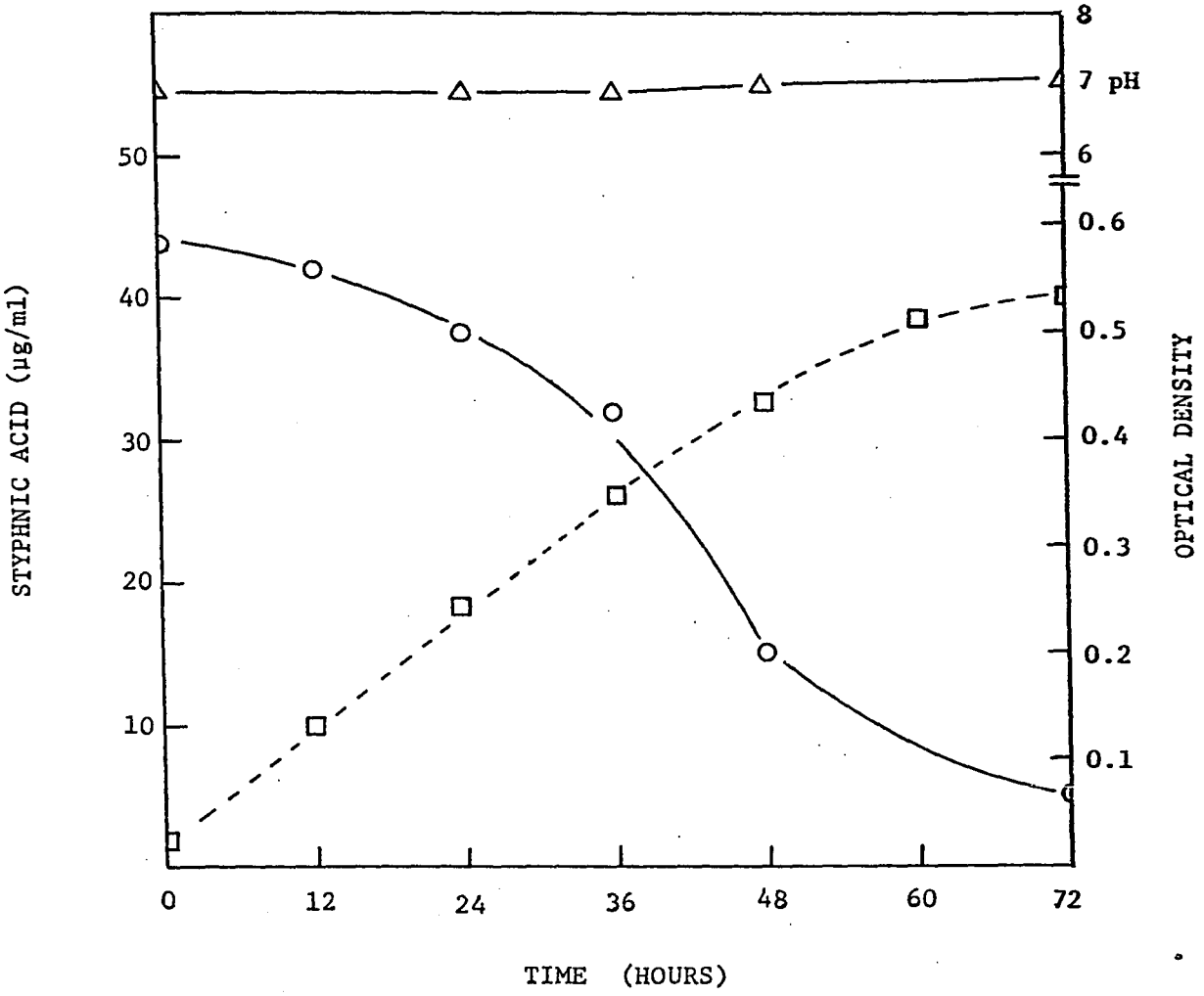

SOLVENT FRONT
1
2
3
1-0
2-0
1-24
2-24
1-72
2-72

Figure 8. Relationship between the growth of the organisms, the pH of the medium and the disappearance of styphnic acid.




STYPHNIC ACID (µg/ml)
50
40
30
20
10
8
7 pH
6
0.6
0.5
0.4
0.3
0.2
0.1
OPTICAL DENSITY
0
12
24
36
48
60
72
TIME (HOURS)

completed as the cells reached the stationary phase. The pH of the medium remained constant at pH 7.0 throughout the experiment.

Hydrolysis of the cells and of the supernatant

Acid and alkaline hydrolysis of the cells and of the supernatant were carried out to demonstrate that styphnic acid did not accumulate in the microbial cells while disappearing from the supernatant and thus to show that styphnic acid was metabolized by the cells. The data in Table 4 show that the supernatant contained 212 µg/ml of styphnic acid at the beginning of the experiment and that after 60 hours, substantial degradation occurred since only 40 µg/ml were left in the medium. After hydrolysis, both alkaline and acid, the treated supernatants contained the same amount of styphnic acid as the untreated supernatants. Furthermore, the concentration of styphnic acid in the treated supernatants decreased with time while there was no accumulation of the substrate in the cells. Controls consisting of uninoculated media were submitted to the same treatment as the samples and the data indicated that the concentration of styphnic acid was unaltered by the hydrolysis.

Thin layer chromatography substantiated this finding. Figure 9 indicates that styphnic acid spots in the supernatant decreased in colour intensity after 60 hours, showing that the substrate concentration was lowered; no styphnic acid was detected in

TABLE 4. Analysis after acid and alkaline hydrolysis.

Spectrophotometric analysis of the supernatant before hydrolysis.

Time (hours)	Styphnic acid (µg/ml)
0	212
24	136
60	40

ACID HYDROLYSIS

Spectrophotometric analysis of the supernatant and of the cells after hydrolysis.

Time (hours)	Styphnic acid (µg/ml)		
	supernatant	cells	control
0	225	3	205
24	120	5	209
60	35	5	215

ALKALINE HYDROLYSIS

Spectrophotometric analysis of the supernatant and of the cells after hydrolysis.

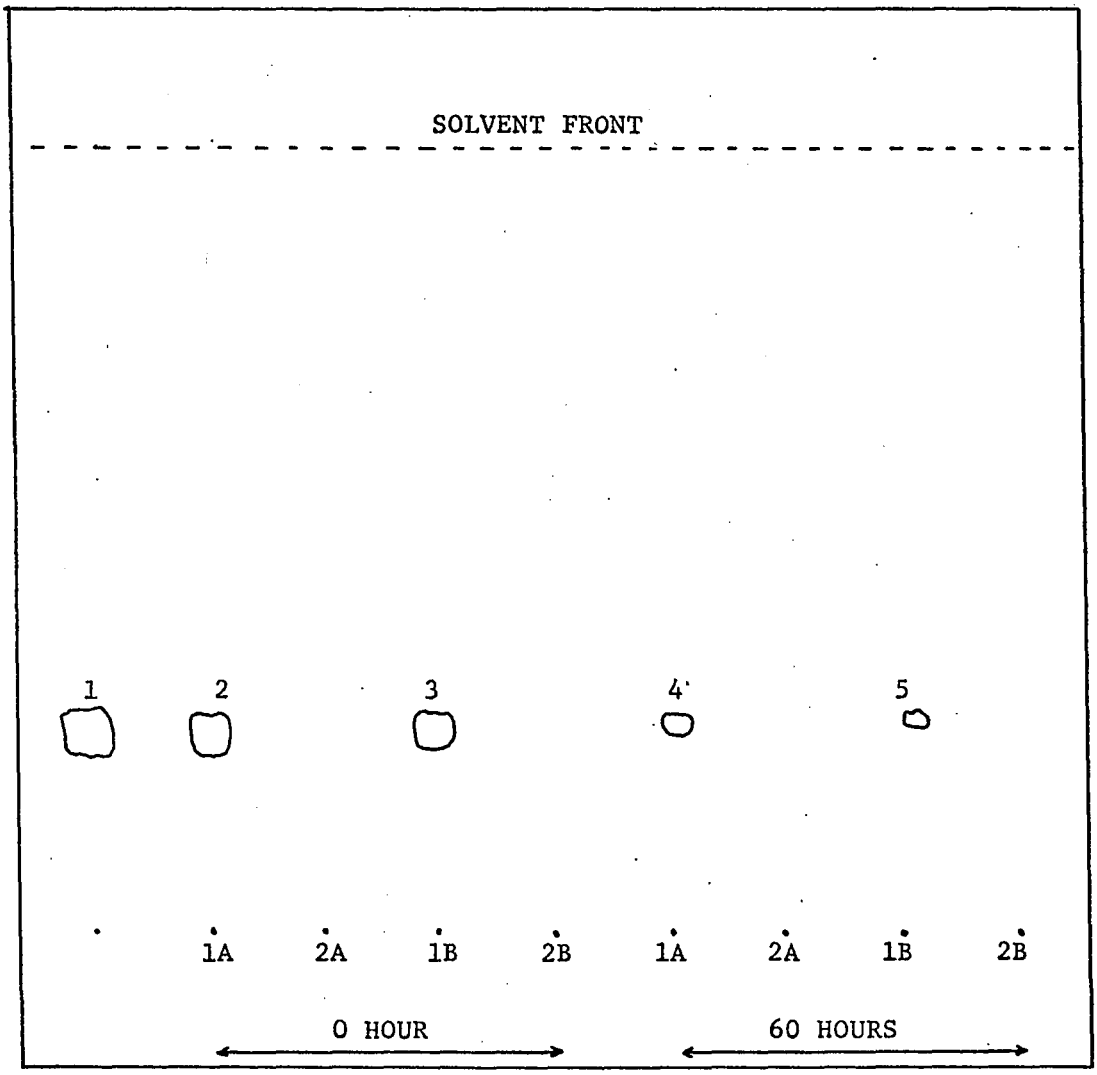
Time (hours)	Styphnic acid (µg/ml)		
	supernatant	cells	control
0	220	5	212
24	118	3	200
60	30	2	208

Figure 9. Chromatogram of ether extracted samples after acid and alkaline hydrolysis.

Sample No.

1A	Acid hydrolysis of the supernatant
2A	Acid hydrolysis of the cells
1B	Alkaline hydrolysis of the supernatant
2B	Alkaline hydrolysis of the cells

Spot No.	Colour	Colour intensity	Rf value	Identity
1	Yellow	++++	0.25	Control styphnic acid
2	"	++++	"	Sample spot
3	"	++++	"	" "
4	"	+	"	" "
5	"	+	"	" "


SOLVENT FRONT
1
2
3
4
5
1A
2A
1B
2B
1A
2A
1B
2B
0 HOUR
60 HOURS

sedimented cells. Thus styphnic acid was degraded and did not accumulate in the cells.

Standardization of the inoculum

An experiment was performed to check the reproducibility of results with a mixed culture. Inocula were grown separately under identical conditions and added to different standard media. Styphnic acid degradation was followed spectrophotometrically in each fermentation vessel and Table 5 shows that the degradation pattern is almost identical. Thus the assumption was made that reproducible results could be obtained with a mixed culture.

Measurement of growth

Figure 10 shows the relationship between the turbidity of the mixed culture in the standard medium and the dry weight of the cells.

FACTORS AFFECTING THE DEGRADATION OF STYPHNIC ACID BY A MIXED CULTURE

Nutrients

The effects of nutrients was assessed by omitting various constituents from the standard medium (phosphate buffer, oligoelements, styphnic acid, and vitamin free casamino acids). All experiments were performed at 25 C.

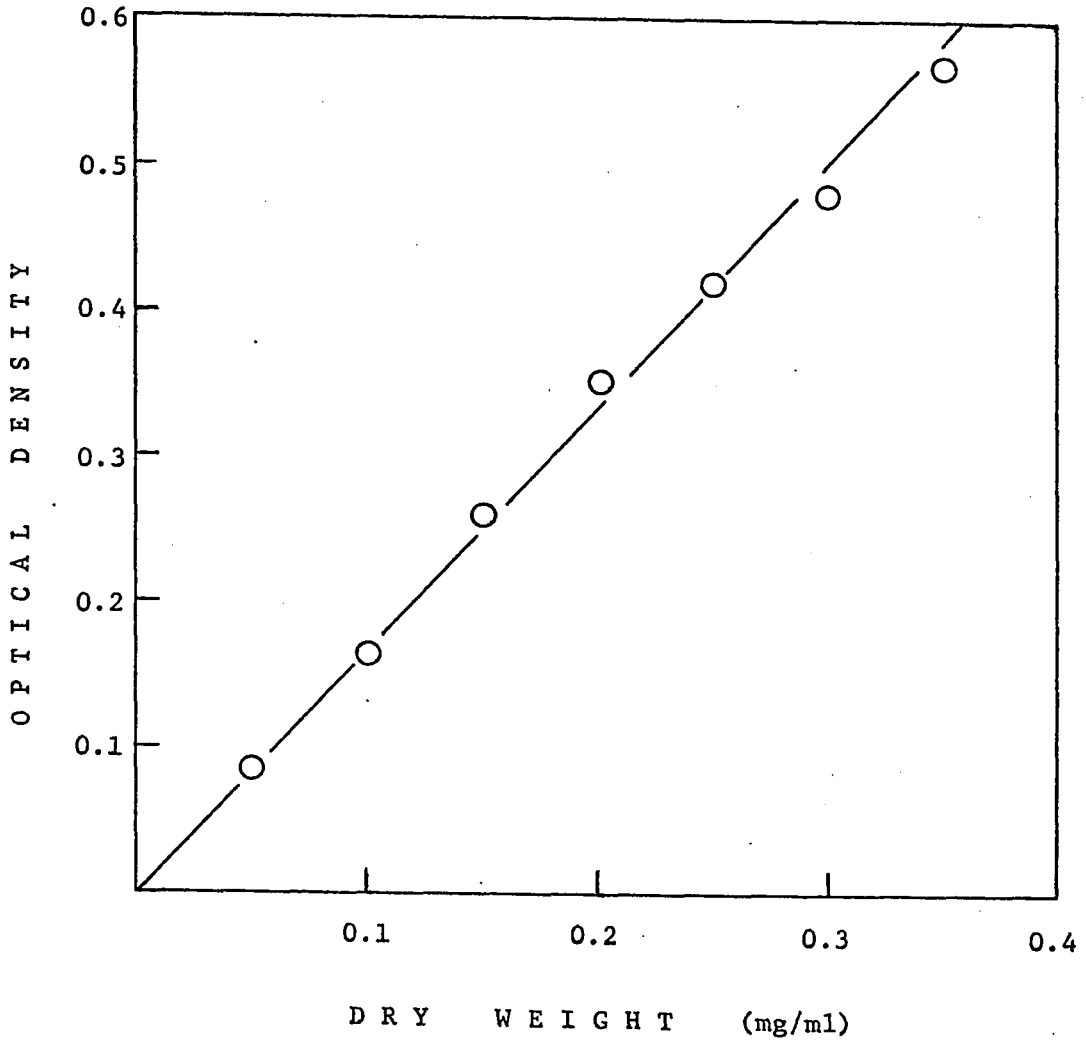
a) Yeast extract and vitamin free casamino acids

Yeast extract was shown to be a satisfactory nutrient source for

TABLE 5. Degradation of styphnic acid by two different inocula at 25 C.

Time (hours)	Styphnic acid (µg/ml)	
	Flask A	Flask B
0	37	37
21	33	33
42	25	21
49	18	14
64	10	8
69	8	7
74	7	6
87	5	5
95	4	4

Figure 10. Relationship between the turbidity and the dry weight of the cells of the mixed culture.

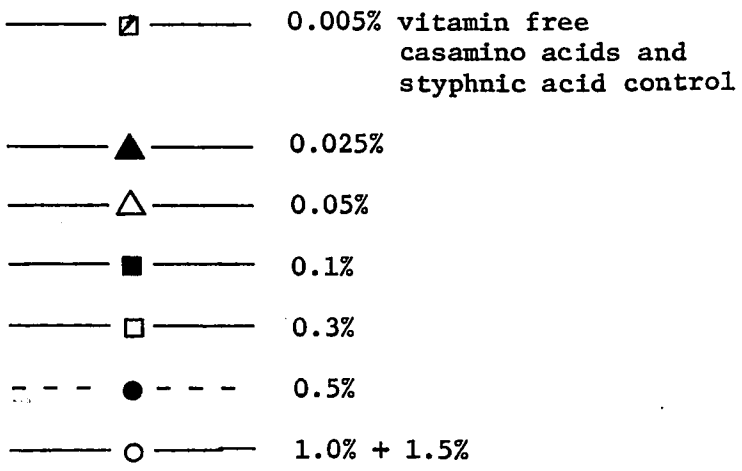

0.6
0.5
0.4
0.3
0.2
0.1
OPTICAL DENSITY
0.1
0.2
0.3
0.4
DRY WEIGHT (mg/ml)

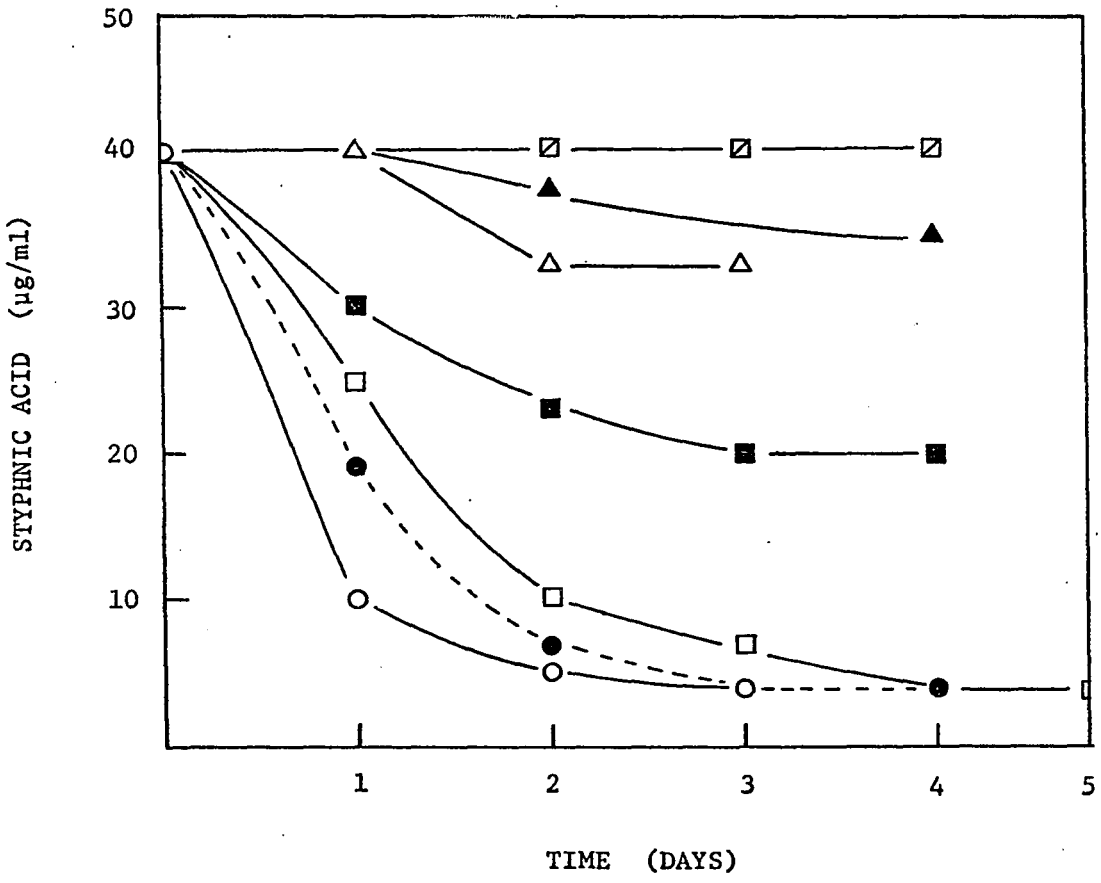
the degradation of styphnic acid (see Table 3). Standard medium, containing vitamin free casamino acids instead of yeast extract, was tested at various concentrations of the casamino acids and Figure 11 illustrates the degradation rate of styphnic acid at various concentrations of the additive. No degradation was found where no casamino acids were added while 100% of the styphnic acid disappeared with 0.3 to 1.0% of the additive. The rate of degradation increased as the concentration of the additive was increased but maximal rate was found at the 1.0% level and above. At concentrations of 0.1% and lower there was only partial degradation of styphnic acid; the less the concentration used, the smaller was the extent of total degradation. Thus casamino acids could replace yeast extract as the added nitrogen source.

b) Tap water versus distilled water

Standard medium was prepared the usual way by adding distilled water and oligoelements while in another set of flasks oligoelements were omitted. Another set of flasks contained tap water which replaced the distilled water and the oligoelements. Table 6 shows that tap water can replace distilled water and the oligoelements and about the same extent and rate of styphnic acid degradation was found as with standard medium. However, with distilled water alone, only 50% degradation was found. The fact that tap water can be used has considerable importance from a practical point of view.

Figure 11. The effect of vitamin free casamino acids on the degradation of styphnic acid.




50
40
30
20
10
STYPHNIC ACID (µg/ml)
1
2
3
4
5
TIME (DAYS)

c) Synthetic amino acid mixture

A synthetic amino acid mixture approximating the amino acid composition of casein was added to standard medium instead of vitamin free casamino acids. Table 6 shows that there was no styphnic acid degradation at any of the concentrations used.

d) The addition of a nitrogen source and of a carbon source

Glucose at a 0.5% level was added to nitrogen sources in a medium containing a phosphate buffer, oligoelements, and styphnic acid. The nitrogen sources were 0.5% ammonium chloride, 0.5% ammonium nitrate, and 0.5% urea. Growth occurred but styphnic acid was not degraded as shown in Table 6.

e) Styphnic acid and a nitrogen source

Glucose was omitted from the medium described in section d). The use of inorganic or organic nitrogen source did not stimulate growth except for urea. Table 6 shows that the level of styphnic acid during the fermentation remained unchanged.

f) Styphnic acid as sole carbon and nitrogen source

Medium containing styphnic acid, phosphate buffer, and oligoelements was seeded with inoculum to check whether the microorganisms would utilize the carbon atoms and the nitrite groups of the styphnic acid molecule as an energy source and as biosynthetic substrate. No

growth occurred, and the styphnic acid concentration did not change during the experiment as shown in Table 6.

Aeration

a) The effect of agitation

Standard medium containing 50 µg/ml of styphnic acid were incubated at 25 C under different conditions: one flask was placed on a rotary shaker at 75 oscillations per minute, one flask was stirred with a 1 inch magnet on a magnetic stirrer at 50 revolutions per minute, sufficient to give slow mixing, and the last flask was left stationary except for occasional shaking on sampling. No degradation of styphnic acid was found with agitation while almost complete utilization occurred in the stationary culture. The flask which was stirred slowly showed a 40% degradation.

b) Relationship between volume and surface area

Different volumes of standard medium were added to 500 ml Erlenmeyer flasks as illustrated in Table 7. The surface area was calculated from the measurement of the diameter of each flask at the level of the different liquid solutions. Each flask had the same concentration of styphnic acid (50 µg/ml) and the same percentage of inoculum. Solutions were filter sterilized and were prepared in duplicate in order to take an initial reading without affecting the ratio of volume to surface area. After inoculation,

TABLE 6. Degradation of styphnic acid related to the addition of various nutrients.

A The addition of oligoelements and tap water

Time (hours)	Distilled water	Distilled water + oligoelements	Tap water
0	50*	50	50
24	48	44	43
48	35	22	25
72	25	7	8
96	25	5	6

B The addition of a synthetic amino acid mixture

Time (hours)	Amino acid mixture				
	2%	1%	0.5%	0.3%	0.1%
0	50*	50	51	50	51
24	51	50	52	53	50
120	50	52	49	50	50

C The addition of a carbon source, a nitrogen source and styphnic acid

Time (hours)	Ammonium chloride + glucose	Ammonium nitrate + glucose	Urea + glucose
0	66*	68	66
10	66	68	68
20	68	68	68
70	68	68	66

* Residual styphnic acid (µg/ml)

TABLE 6. (continued)

D The addition of a nitrogen source to styphnic acid

Time (hours)	Ammonium chloride	Ammonium nitrate	Urea
0	66*	66	68
10	68	66	68
20	68	68	68
70	68	68	68

* Residual styphnic acid (µg/ml)

E Styphnic acid as sole carbon and nitrogen source

Time (hours)	Residual styphnic acid (µg/ml)
0	66
24	64
48	66
60	68
72	66

the flasks were incubated without agitation at 25 C for 3 days.

Optimal degradation occurred between a ratio of volume to surface area of 6 to 1.3 as shown in Table 8. At higher ratios the degradation was partial but degradation was even less complete when the cultures were well aerated. Thus the degradation of styphnic acid is maximal when there is a limited supply of oxygen.

Temperature

Standard medium was inoculated and incubated at various temperatures controlled ± 1 C. The control contained no vitamin free casamino acid and there was no degradation in the control and none at 45 C, 20% at 10 and 40 C with complete degradation at 15, 20, 25, 30, and 35 C. The rate of degradation, as illustrated in Figure 12, increased with increasing temperature up to the optimal temperature at 35 C. As the temperature was increased above optimum there was a sharp decrease in degradation.

The efficiency of the system was evaluated by calculating the percentage degradation of styphnic acid for each temperature tested after 60 hours, which represents the maximal degradation rate of styphnic acid at 35 C. The results are illustrated in Figure 13. Maximal efficiency was obtained at 35 C. Even though complete degradation was obtained at 15, 20, 25, 30, and 35 C, there is a marked difference in efficiency when the time factor is considered: at 15 C the efficiency is below 5% while at 35 C the efficiency is ~~16 fold.~~ SIXTEENFOLD

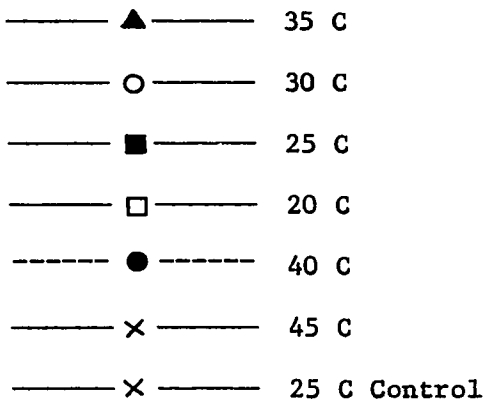
TABLE 7. Protocol for experiment showing the relationship between volume and surface area.

Size of flask	Total volume	Distilled water	Inoculum	0.25% Styphnic acid
ml	ml	ml	ml	ml
500	500	485	5	10
500	300	291	3	6
500	100	97	1	2
500	50	48.5	0.5	1
500	25	24.25	0.25	0.5

TABLE 8. The relationship between volume and surface area on the degradation of styphnic acid.

Volume of medium	Volume/ surface area	Initial styphnic acid concentration	Final styphnic acid concentration	% Degradation
ml	(ml/cm^2)	(µg/ml)	(µg/ml)	
500	40	50	15	70
300	6	50	7	86
100	1.3	50	9	82
50	0.6	50	23	54
25	0.4	50	32	36

Figure 12. The degradation of styphnic acid at different temperatures.



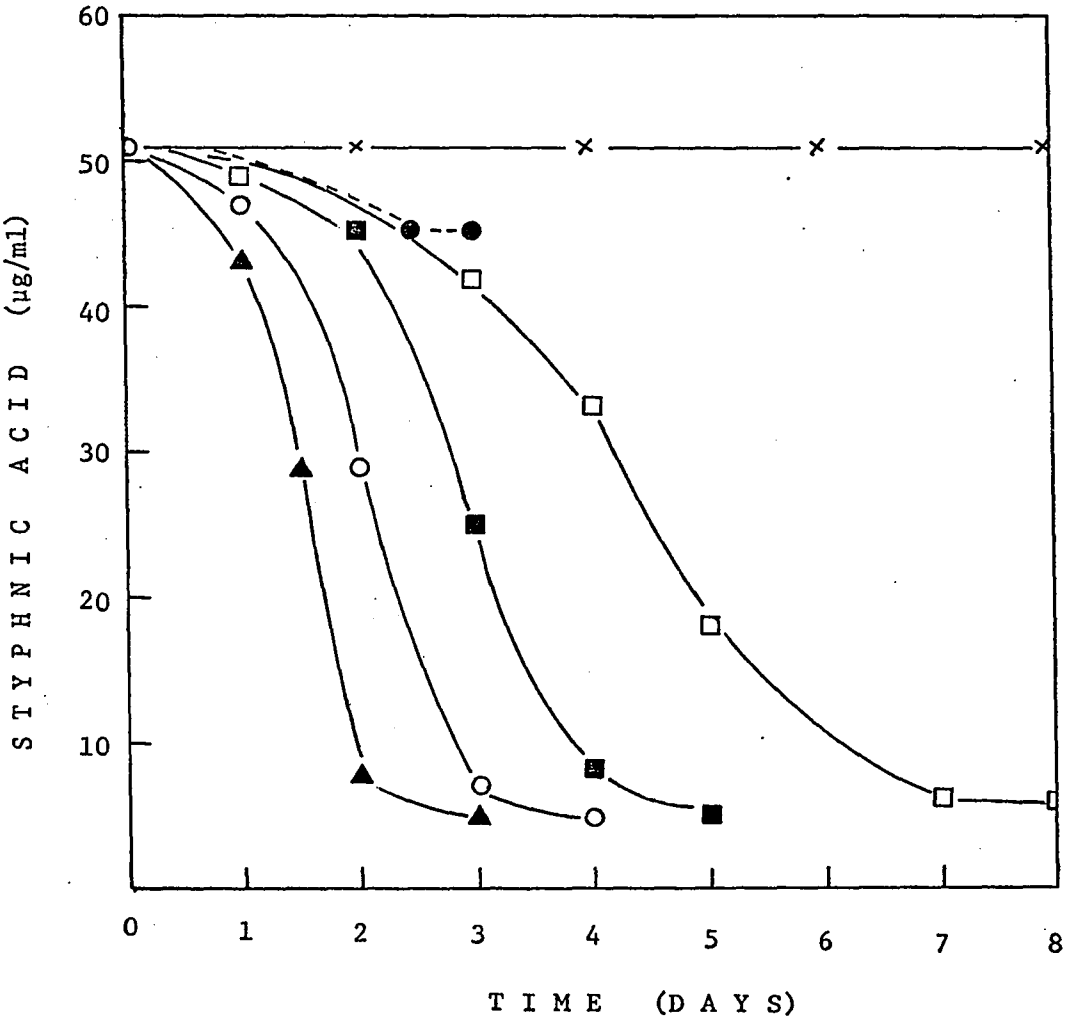
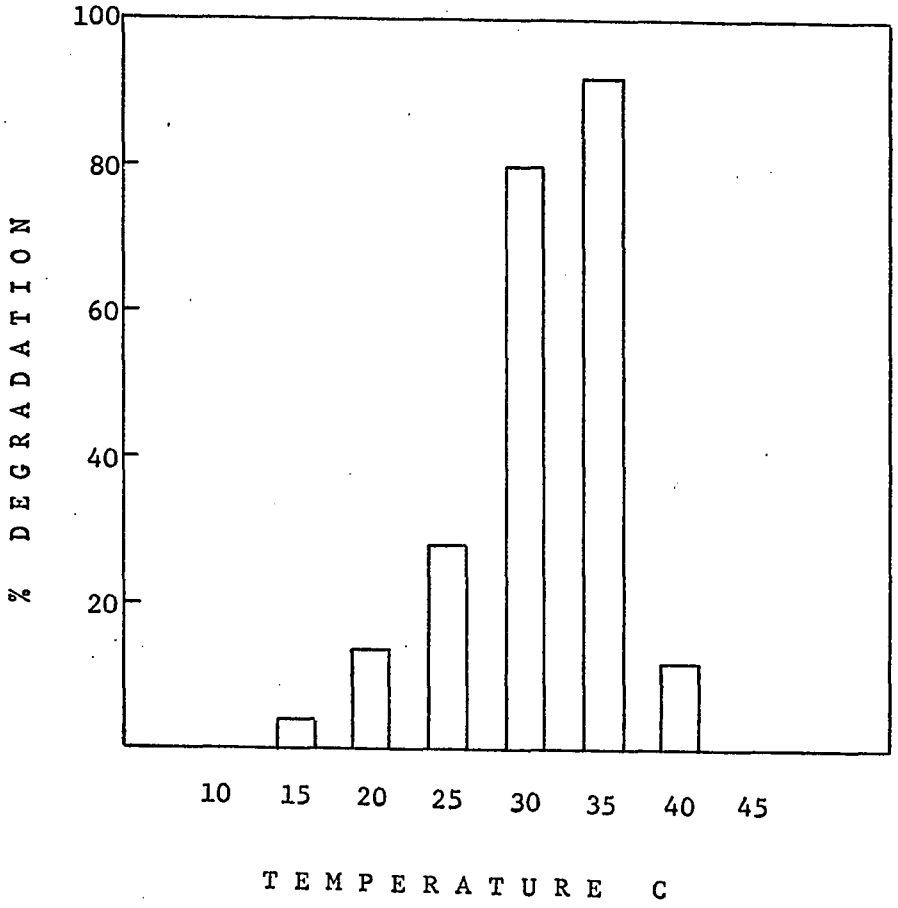

60
50
40
30
20
10
STYPHNIC ACID (µg/ml)
0
1
2
3
4
5
6
7
8
TIME (DAYS)

Figure 13. The percentage of degradation of styphnic acid at different temperatures.


100
80
60
40
20
% DEGRADATION
10
15
20
25
30
35
40
45
TEMPERATURE C

This phenomenon is of considerable importance from a practical point of view.

pH

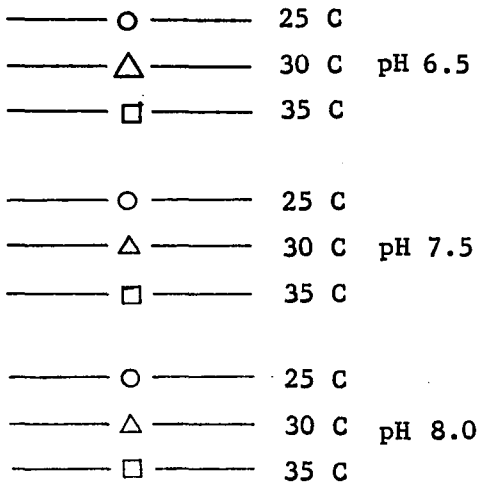
The standard medium was buffered with 0.05 M of a phosphate buffer for pH 6.0, 6.5, and 7.5, of a Tris buffer for pH 8.0, and of a glycine-NaOH buffer for pH 9.0 and 10.0. The solutions were filter sterilized, and the inoculum was resuspended in each appropriate buffer before its addition to the different flasks. The intial pH was unchanged throughout the experiment. When the incubation temperature was 25 C, there was no degradation at pH 6.0, 9.0, and 10.0; at 30 and 35 C, there was no degradation at pH 6.0 and 10.0 but there was complete degradation of styphnic acid from pH 6.5 to 9.0.

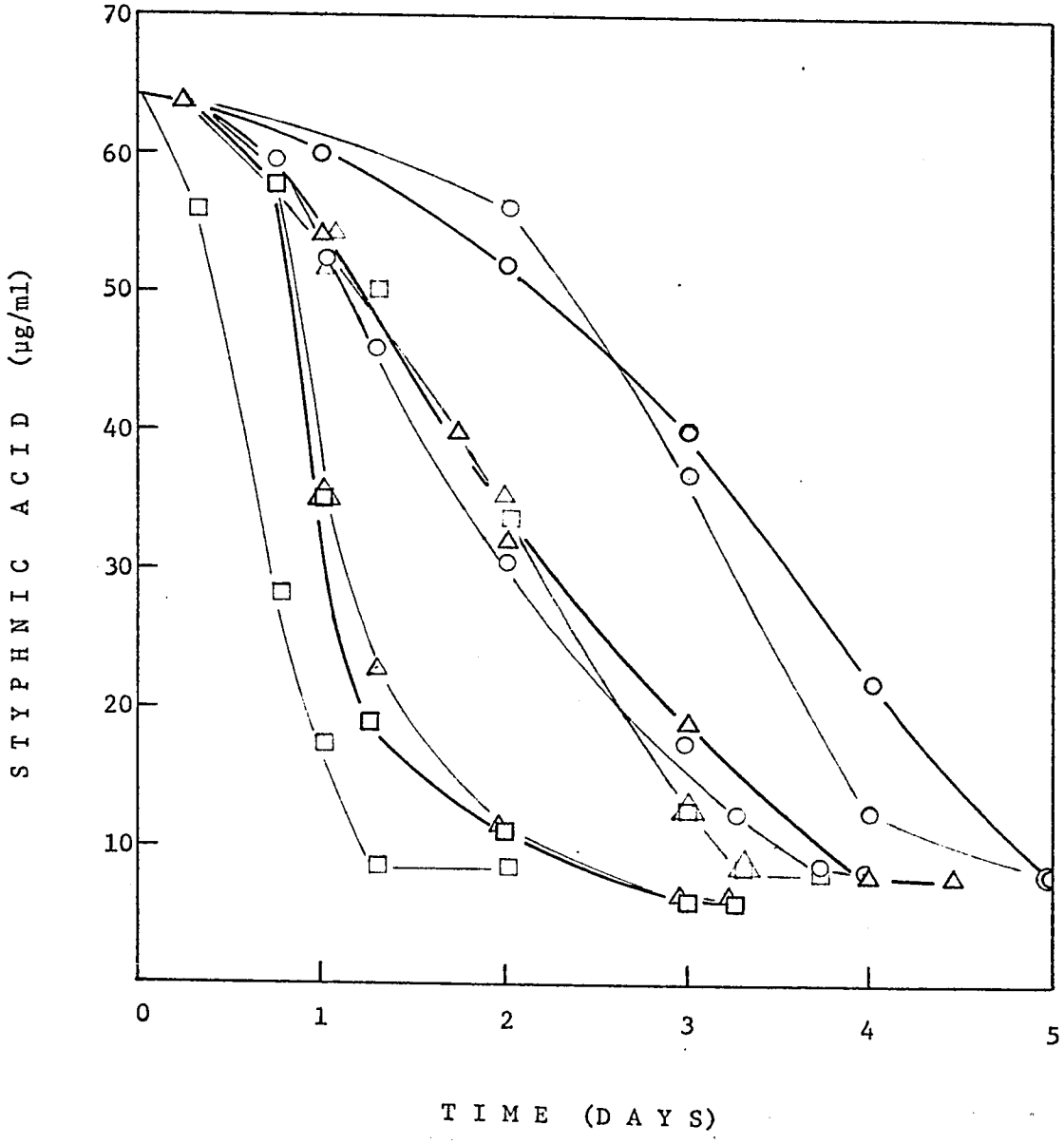
Figure 14 shows the relationship between the rate of degradation of styphnic acid at different pH values and the temperature of incubation. The rate of degradation of styphnic acid is similar from pH 6.5 to pH 8.0 at 25 C; at pH 8.0 at 30 and 35 C, pH 6.5 at 30 C, and pH 7.5 at 25 C; and between pH 7.5 at 30 C and pH 6.5 at 35 C.

Styphnic acid concentration

Different concentrations of styphnic acid were obtained by adding various amounts of 0.2% stock solution to standard medium with the pH adjusted to 7.5 before autoclaving. The control consisted of uninoculated medium at pH 7.5. Results of styphnic acid degradation over

Figure 14. The disappearance of styphnic acid at pH 6.5, 7.5 and 8.0 at various temperatures.




70
60
50
40
30
20
10
0
1
2
3
4
5
STYPHNIC ACID (µg/ml)
TIME (DAYS)

a wide range of concentrations are shown in Table 9. There was over 70% degradation from 50 - 170 µg/ml range of styphnic acid while at higher concentrations there was only a partial degradation.

Figure 15 shows that at each level of substrate used, 50,100, and 150 µg/ml, the rate of degradation was similar at any temperature although the extent of degradation of styphnic acid was not as complete when the substrate concentration was 150 µg/ml.

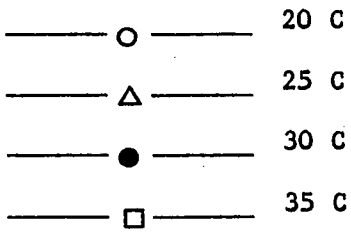
The use of commercial nutrients

Different commercial nutrients such as sulphite liquor, malt extract, and corn steep liquor were tested as additives to replace vitamin free casamino acids. Table 10 shows that sulphite liquor did not bring about any degradation of styphnic acid, even though the concentration of styphnic acid added was very low. The addition of corn steep liquor did not stimulate any degradation. Better results were obtained with malt extract. There was a 50% degradation using 1% malt extract. At higher concentration there was no degradation while at lower concentration there was very little degradation. The initial pH remained unchanged throughout the experiment. Corn steep liquor was used more successfully as shown in Figure 16. Complete and fast rates of degradation were obtained by using 10%, 5%, and 3% of this additive. There was a 60% degradation at 1% and very little degradation at 0.5% level. Standard medium with added corn steep liquor replacing vitamin free casamino acids was modified by

TABLE 9. The rate of degradation of styphnic acid at various concentrations at 25 C.

Time (hours)	Residual styphnic acid concentration (µg/ml)										
											Control
0	57	113	169	224	280	330	392	432	460	555	114
17	50	104	157	216	268	314	378	436	460	535	115
37	32	88	133	189	238	288	348	408	455	500	116
59	11	42	75	115	158	190	232	272	320	400	---
107	7	14	47	85	120	152	202	248	295	350	114
131	7	14	43	80	115	137	195	240	295	330	118

Figure 15. The effect of various styphnic acid concentrations at different temperatures.



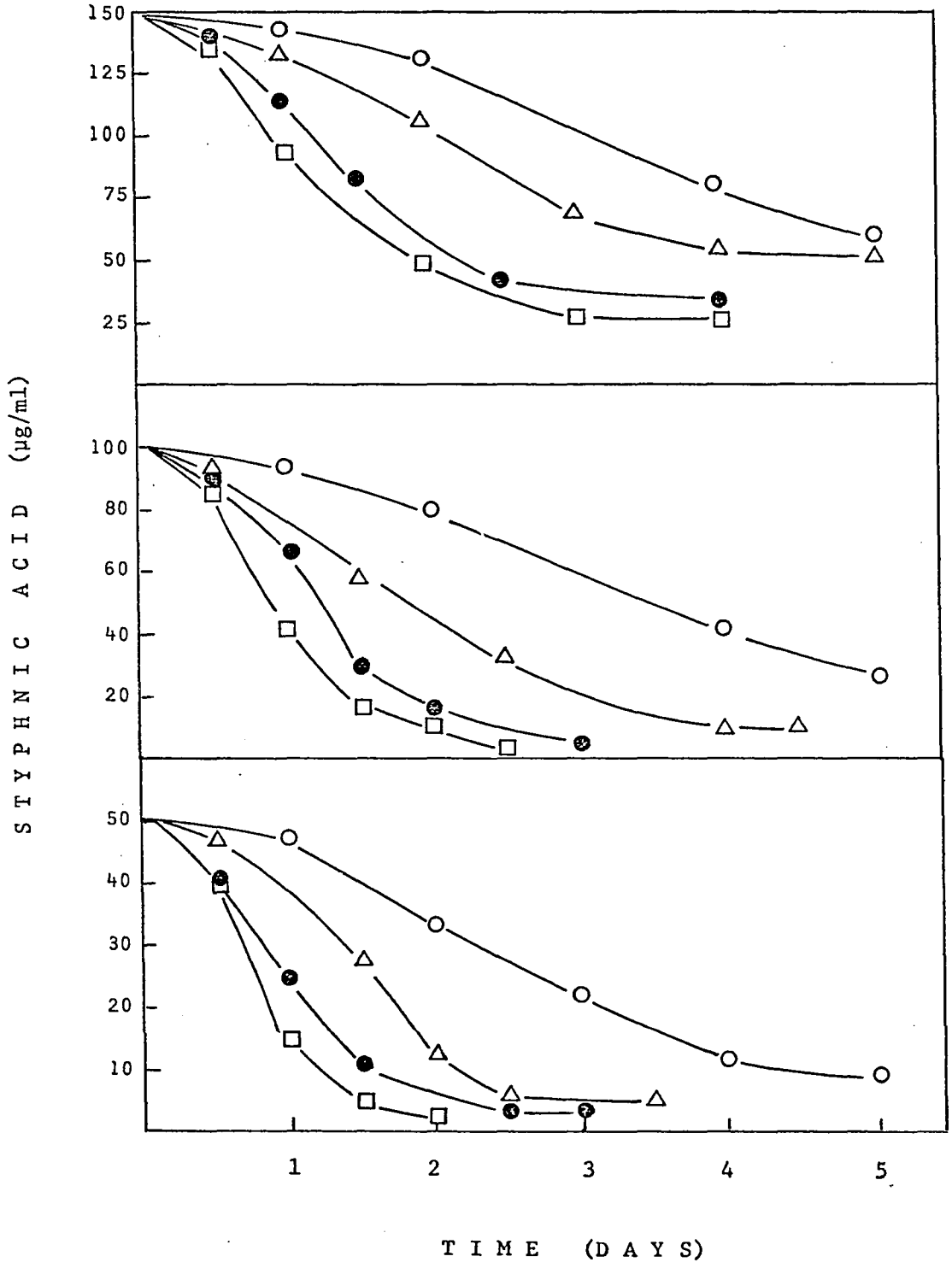

150
125
100
75
50
25
100
80
60
40
20
50
40
30
20
10
1
2
3
4
5
STYPHNIC ACID (µg/ml)
TIME (DAYS)

TABLE 10. The use of sulphite liquor and malt extract as additives in the degradation of styphnic acid.

SULPHITE LIQUOR:

Time (hours)	Dilution of the liquor			
	2:1	1:1	1:2	1:2 + 0.5% corn steep liquor
0	33*	35	35	32
24	33	35	35	30
48	--	--	--	--
72	32	35	34	28
120	32	35	34	28

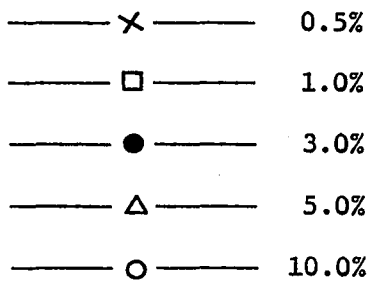
* Residual styphnic acid (µg/ml)

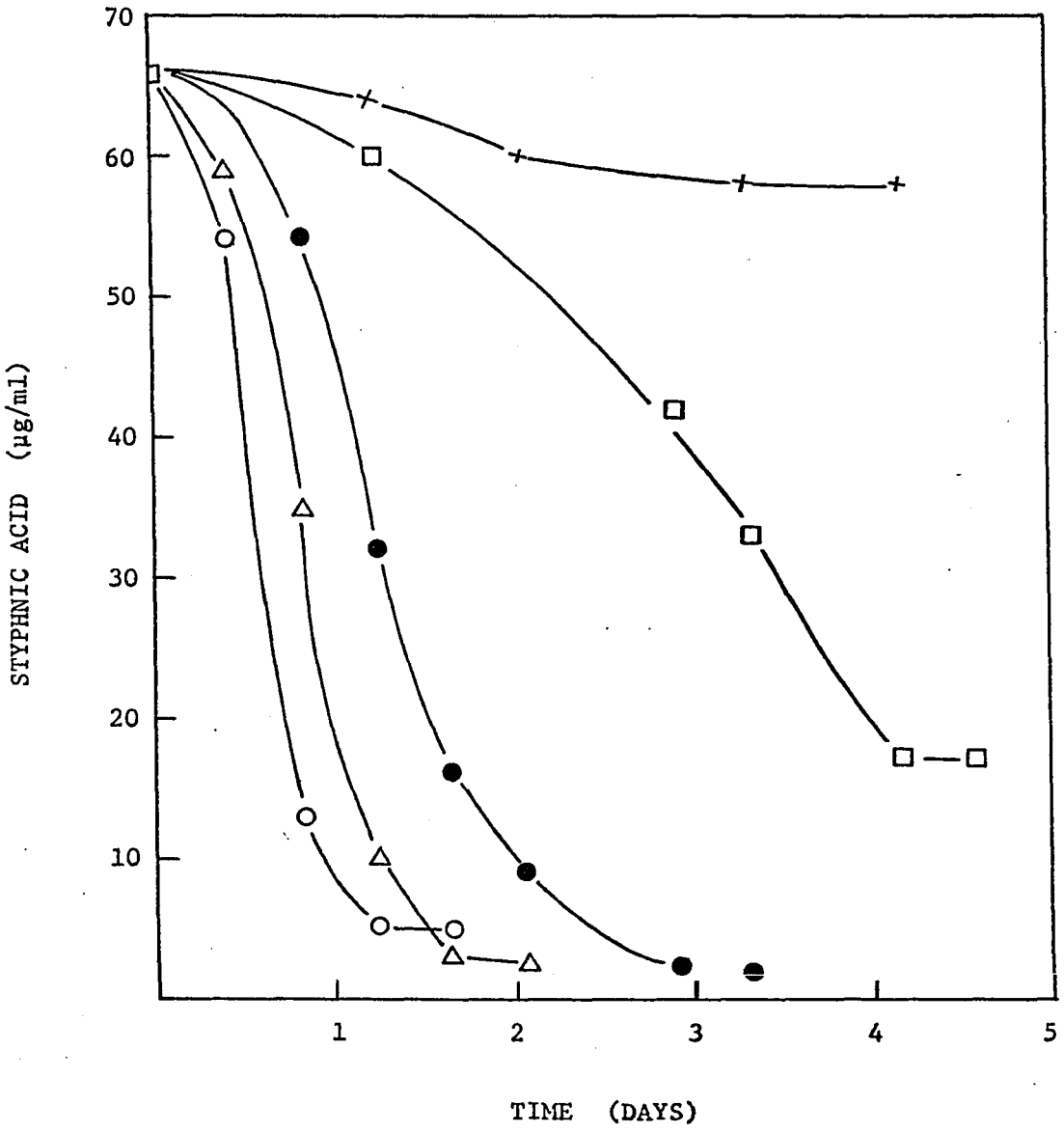
MALT EXTRACT:

Time (hours)	Malt extract			
	3%	1%	0.5%	1% (control)
0	54*	59	62	0
24	54	56	59	0
48	52	48	54	0
72	50	36	51	0
120	42	20	48	0
144	42	20	48	0

* Residual styphnic acid (µg/ml)

Figure 16. The effect of corn steep liquor on the disappearance of styphnic acid.




70
60
50
40
30
20
10
STYPHNIC ACID (µg/ml)
1
2
3
4
5
TIME (DAYS)

omitting the oligoelements and by using tap water. Similar rates and amount of degradation was obtained.

PRODUCTS OF STYPHNIC ACID DEGRADATION

Ammonia vapours were not detected in the atmosphere in which the organism degrading styphnic acid were growing. No free nitrite was detected in the medium. No intermediates were detected with thin layer chromatography by spraying with VTPSA which is specific for phenols. The measurement of oxygen uptake using standard respiratory technique was carried out without success. If cells were added to different concentrations of styphnic acid, there was strong endogenous respiration but no substrate degradation. If vitamin free casamino acid was added, even in trace amount, to the system, degradation occurred but the respiratory activity was very high and could not be dissociated from the endogenous activity.

A brownish colour was produced in the standard medium during degradation of styphnic acid as shown in Figure 6. The brownish material was collected by centrifugation of the cells and the following tests for melanin pigments were negative (Hackman, 1953); boiling with ethanolic KOH; heating with concentrated KOH; and reduction with sodium dithionite followed by decolorization in potassium permanganate. The brownish material is not a humic acid since it is not soluble in 0.5 N NaOH. Microscopic examination indicated the material was incorporated in the cells.

DEGRADATION OF VARIOUS NITROPHENOLS BY THE MIXED CULTURE

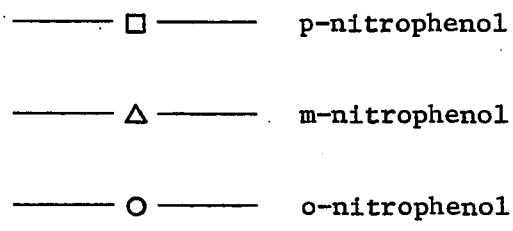
The mixed culture was tested for its ability to degrade a variety of other nitrophenols. Figure 17 shows that o-, m-, and p-nitrophenol were degraded to a large extent and while the attack was rapid on o-nitrophenol, it was slower on m- and p-nitrophenol. Figure 18 shows that 2,4-dinitrosoresorcinol and 2-nitroresorcinol were attacked as rapidly as styphnic acid but that the degradation was only 50% complete. The compounds 2,4-dinitrophenol and 2,4,6-trinitrophenol (picric acid) were degraded over 60% but the rate of attack was slower.

Thin layer chromatography also substantiated the disappearance of the compounds as shown above. Figure 19 shows that m-nitrophenol disappeared after 72 hours, that p-nitrophenol was not completely metabolized after 120 hours but that its concentration had been lowered and that 2,4-dinitrophenol and 2,4,6-trinitrophenol were metabolized to different extents; with the latter substrate other spots were detected but not characterized.

DEGRADATION OF STYPHNIC ACID IN A CONTINUOUS CULTURE SYSTEM

Standard medium containing 50 µg/ml of styphnic acid was added to the fermentation vessels and inoculated with the mixed culture. The mixed culture was allowed to grow until the medium became reddish (when the residual styphnic acid was about 15 - 20 µg/ml), which is

Figure 17. The degradation of various nitrophenols by the mixed culture.



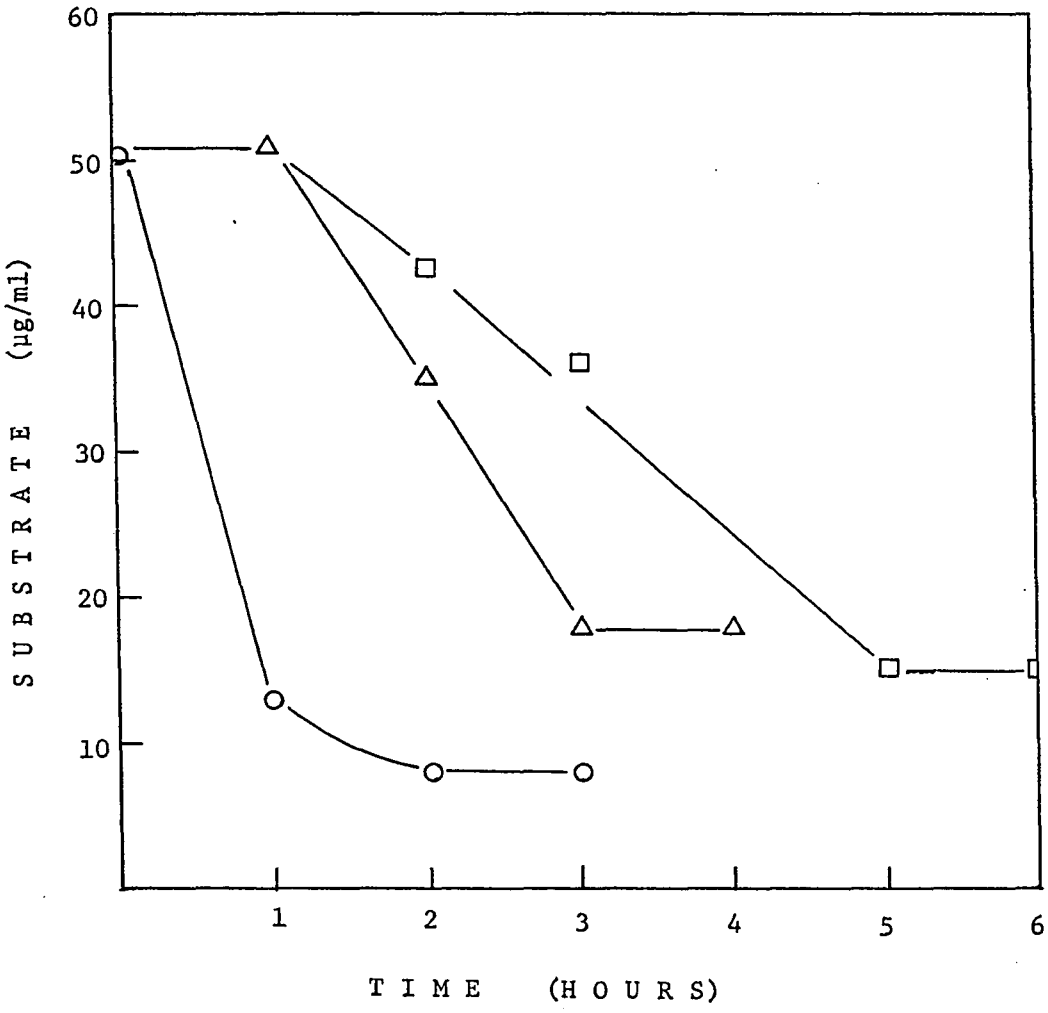
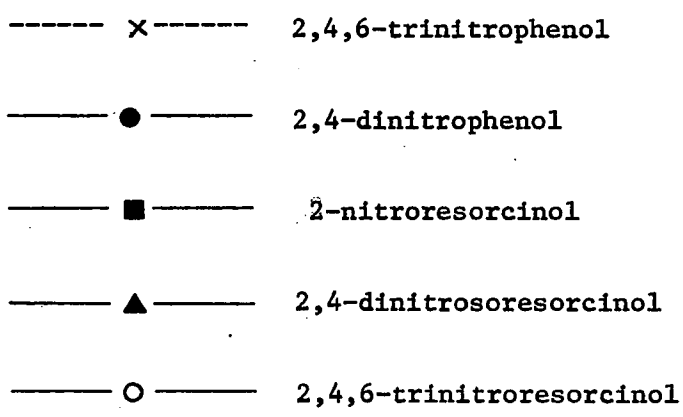

60
50
40
30
20
10
SUBSTRATE (µg/ml)
1
2
3
4
5
6
TIME (HOURS)

Figure 18. The degradation of various nitrophenols by the mixed culture.



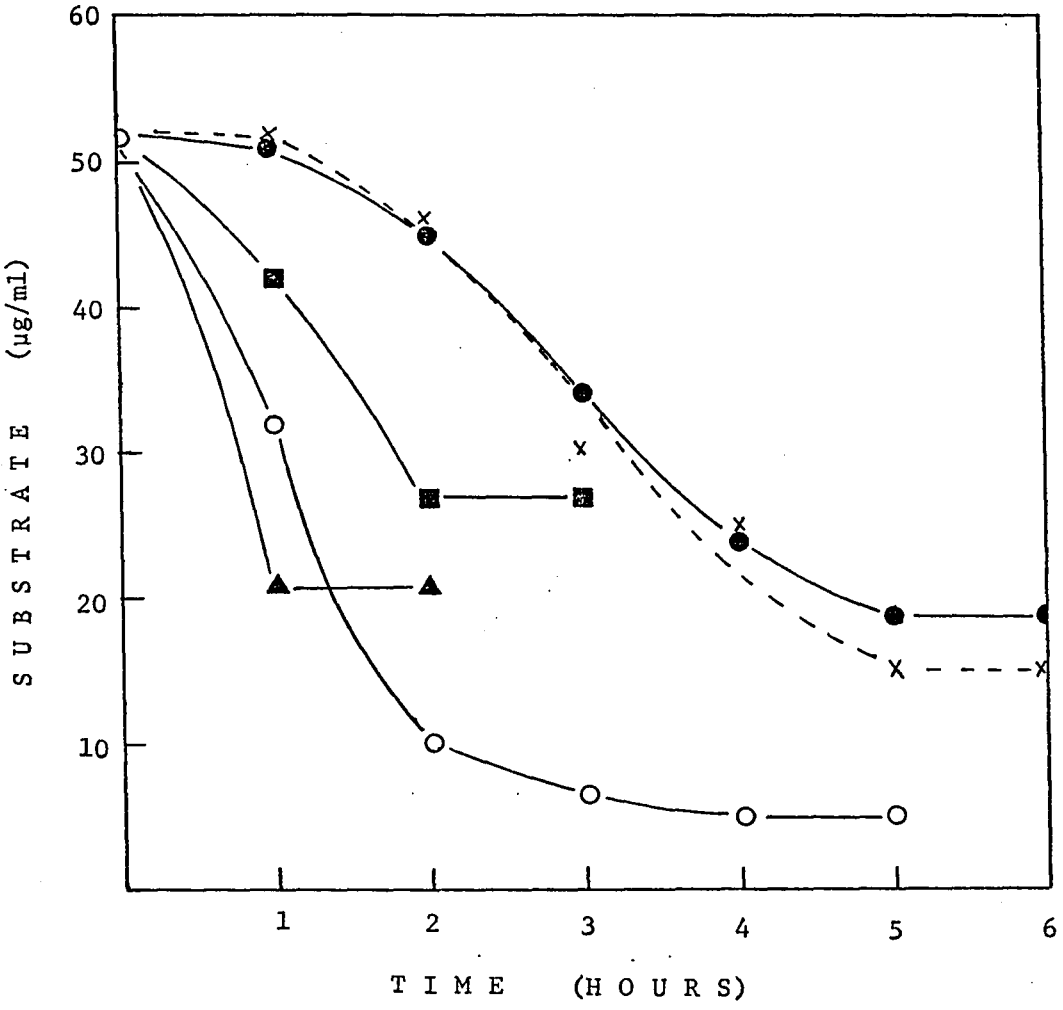

60
50
40
30
20
10
SUBSTRATE (µg/ml)
1
2
3
4
5
6
TIME (HOURS)

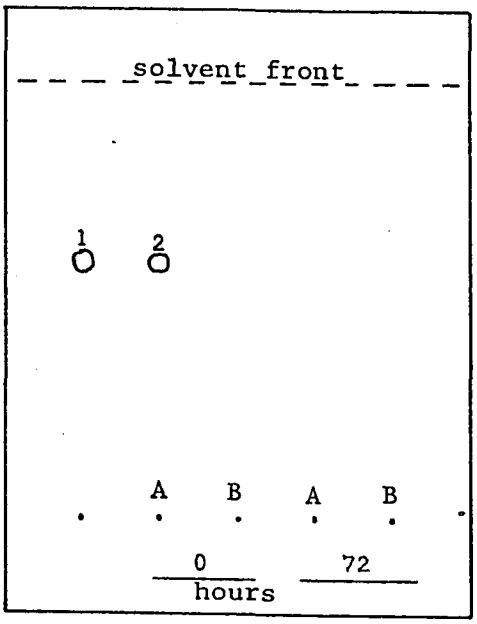
Figure 19. Chromatograms showing the disappearance of various nitrophenols.

A. material from the ether fraction after extraction

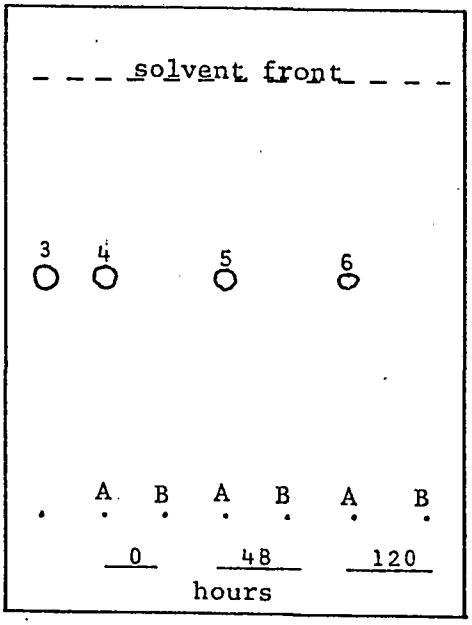
B. material from the water layer after extraction

Spot No.	R_f	Colour	Colour intensity	Identity
1	0.72	Yellow	++	m-nitrophenol control
2	0.72	"	++	sample spot
3	0.69	Yellow	++++	p-nitrophenol control
4	0.69	"	++++	sample spot
5	0.69	"	++++	" "
6	0.69	"	++	" "
7	0.70	Yellow	++++	2,4-dinitrophenol control
8	0.70	"	+++	sample spot
9	0.70	"	++	" "
10	0.70	"	+	" "
11	0.47	Orange yellow	+	" "
12	0.47	" "	+++	" "
13	0.47	" "	++	" "
14	0.76	Yellow	++++	picric acid control
15	0.76	"	++++	sample spot
16	0.76	"	++	" "
17	0.66	Orange	+++	" "
18	0.66	"	+	" "

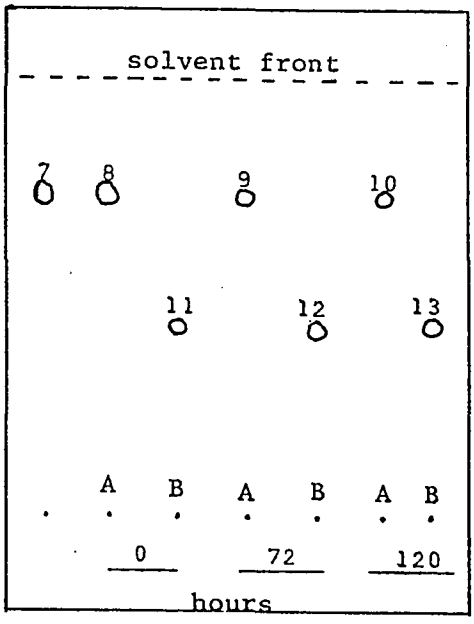
m-nitrophenol



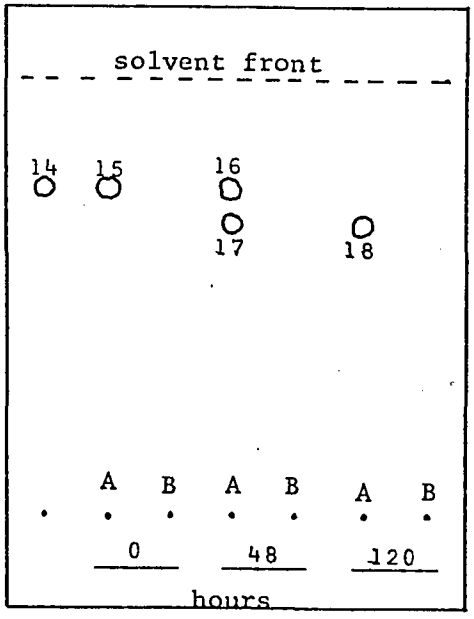
p-nitrophenol



2,4-dinitrophenol



Picric acid



the period of fastest styphnic acid degradation (Figure 8). At this time standard medium with 100 µg/ml of styphnic acid was added at a constant rate to the fermentation flask. Different rates of addition were tested to find which rate of dilution allows the cells to maintain a maximum rate of degradation. The results are tabulated in Table 11. At the fastest dilution rate, the concentration of substrate in the fermentation flasks remained high and 60 hours were necessary for 15,000 µg of substrate to be metabolized. At a slow dilution rate the concentration of substrate in the fermentation flasks was low and 50 hours were necessary for the degradation of 15,000 µg of styphnic acid. At the intermediate dilution rate of 8 ml/hour, the concentration of styphnic acid in the fermentation flasks was about 18 µg/ml and the cultures were of a reddish colour. Only 24 hours were necessary to degrade 15,000 µg of styphnic acid.

The mixed culture was found previously to metabolize 15,000 µg of styphnic acid (300 ml of standard medium containing 50 µg/ml of styphnic acid) in 60 hours under batch conditions. The use of continuous culture improved the efficiency of the system by over 50%.

TABLE 11. Degradation of styphnic acid under continuous culture conditions.

Rate of flow (ml/hr)	Residual styphnic acid (µg/ml)						Time (hours) required to metabolize 15,000 µg of substrate
	Top flask			Bottom flask			
	Time of fermentation (hours)						
	0	24	48	0	24	48	
3	10	12	13	11	13	10	50
8	18	17	--	15	18	--	24
12	35	38	37	34	37	36	60

DISCUSSION

The purpose of this project was a study of the factors affecting the degradation of a nitrophenol, styphnic acid to elucidate possibly the mechanism of the degradation of this compound, and from a practical point of view to provide data for evaluating the possibility of using biological degradation treatment of this compound instead of the chemical treatment used presently. Microbial degradation of styphnic acid was obtained through the isolation of a mixed culture of microorganisms, containing some *Bacillus* species, from a forest soil. Surprisingly, no isolate capable of styphnic acid degradation was obtained from soils previously treated with a nitro-compound or from sand treated with phenolic residues from a petroleum refinery. *Bacillus* species from industrial effluents, rivers, mud, and soil (Cabridenc, 1966), and *Micromonospora* strains from lake mud (Erickson, 1941) were also reported to utilize styphnic acid. Erickson (1941) reported only the growth of *Micromonospora* on unspecified concentrations of styphnic acid as sole carbon source while Cabridenc (1966) obtained a 7% biodegradation of styphnic acid as sole carbon source over a 48 hour period. This study reports 90% biodegradation of styphnic acid under ~~maximum~~ optimal conditions over a 60 hour period in a complex medium. None of the six pure isolates obtained was able to metabolize styphnic acid as sole carbon source even though growth occurred in its presence. A similar phenomenon

was noted by Cabridenc (1966) where *Micrococcus* and *Bacillus* strains would tolerate 2,000 ppm of phenol but did not metabolize the substrate. The degradation of styphnic acid was obtained only with a mixed culture indicating the occurrence of a synergistic effect where one organism required the metabolites of other strains in order to metabolize the substrate. A mixed culture has the advantage of being more representative of an ecosystem since the physiological limitations of individual isolates are avoided. The mixed culture was subcultured for over two years and yielded identical fermentation results, indicating its stability, which contradicts the work of Cabridenc (1966) where a mixed culture of microorganisms was added to an activated sludge system containing a mixture of nitrophenols; he obtained a selection of microorganisms which changed the metabolizing power of the original mixture. This was avoided in this study since only one nitrophenol source was added and the mixed culture was cultivated consistently in the same medium. It was not possible to follow the relative composition of the microflora during the degradation of styphnic acid since none of the isolates or no combination of microorganisms could be identified as responsible for the biodegradation of the substrate. A mixed culture has the potential to attack a variety of other nitro compounds and indeed the microorganisms metabolized o-, m-, and p-nitrophenol, 2,4-dinitrophenol, and picric acid. Gundersen and Jensen (1956) reported similar results with *Arthrobacter*

simplex. They concluded that there was a structural relationship among those nitro-compounds degraded by their organisms as all possessed a NO_2 group in a para position to a hydroxyl group on the benzene ring; two exceptions were noted, 2,4-dinitrophenol and 3,5-dinitrosalicylic acid were not degraded by the organisms. Results obtained from this work suggest that this phenomenon is far from being general since o-, and m-nitrophenol are metabolized and at a faster rate than p-nitrophenol. The mixed culture can thus attack randomly different nitrophenols. Cabridenc (1966) also reported the biodegradation of the above compounds, but at a much slower rate.

The disappearance of styphnic acid was followed by spectrophotometry which was correlated with thin layer chromatography. Similarly Alexander and Lustigman (1966) used a spectrophotometric method but regarded the loss of ultraviolet absorbancy as evidence for the cleavage of the benzene ring. The assay of styphnic acid by spectrophotometry is a faster analytical method than titration with titanium chloride as used by Cabridenc (1966).

Usually low concentrations of these substrates are used and accumulation inside the bacteria has not been investigated. In these studies styphnic acid was shown not to accumulate inside the cells as a result of an uptake, a fact that nobody has shown before.

There is no report in the literature involving the factors affecting the degradation of styphnic acid. Alexander and Lustigman (1966) surveyed the degradation of various nitrobenzenes in soils without controlling pH, temperature, nutrients, etc. Cabridenc (1966) studied from a practical point of view, the degradation of styphnic acid but did not investigate optimum conditions to give maximal degradation of the substrate. The mixed culture used in this study degraded styphnic acid in a complex medium containing either yeast extract, vitamin free casamino acids, or corn steep liquor. A synthetic mixture of amino acids did not replace vitamin free casamino acid even though care was taken to use L-form and amino acids free of acid. Similarly Gundersen and Jensen (1956) added up to 0.1% yeast extract in order to obtain the degradation of dinitro-ortho-cresol while Jensen and Lautrup-Larsen (1967) added various enrichments such as yeast extract, soil extract, and vitamins to their medium. Cabridenc (1966) used a medium containing soil extracts. The concentrations of additives used in this study were higher than those reported by others and undoubtedly were a major factor in the higher rates of degradation observed. Different commercial nutrients were tested in order to find a suitable source of nutrients to replace vitamin free casamino acids. Sulphite liquor and malt extract were not satisfactory even though these nutrients contain carbohydrate materials. Corn steep liquor has been used extensively in

microbiology (Liggett and Koffler, 1948). It contains mineral nutrients, reducing sugars, lactic acid, amino acids, and B-complex vitamins with the exception of thiamine which is low or absent. On an industrial scale the degradation of styphnic acid could be carried out in a medium containing corn steep liquor and tap water, omitting the trace elements and buffer that must be usually added to a chemically defined medium. In a practical trial the mixed culture was tested for its potential to degrade styphnic acid in a continuous culture system. Results from this work suggest that the degradation of styphnic acid by a continuous culture can be done faster than with batch cultures. In a comparable study, Evans and Kite (1962) tested a homogeneous continuous culture to the treatment of spent liquor from cokeries. The spent liquor contained thiosulphates, sulphite, chloride, cyanide, monohydric and polyhydric phenols. Phenol was the limiting substrate and was present in the culture in low concentrations which did not inhibit the bacteria. They developed a mixed population of micro-organisms to metabolize the maximum number of diverse phenolic compounds found in the liquor and obtained 90% removal up to at least 20 g per litre of culture per day with retention times from 1.5 to 2.5 hours. The mixed culture in this study has the potential of degrading a variety of nitrophenols as was discussed previously but no experiment was carried out to see how the mixed cultures would react towards a mixture of nitrophenols. However, such an

experiment was carried out by Cabridenc (1966): a mixed culture was added to a laboratory model of an activated sludge system. The effluent used consisted of a mixture of phenolic compounds approximating the composition of an effluent from an industry manufacturing explosives. There was a marked loss of BOD_5 but the chemical oxygen demand and the phenol concentration were only partially reduced. Chromatographic studies showed that phenols, cresols, p-chlorophenol, resorcinol, and p-nitrophenol were metabolized but that di-substituted compounds and most of all tri-substituted compounds were little metabolized and this may be a drawback of the system from a practical point of view. Several advantages show up from this study besides the potential of the mixed culture to degrade a variety of nitro-compounds. A continuous culture system would be much smaller in size than an activated sludge system or a percolating filter. Aeration is often stated as the most expensive running cost for an activated sludge system; in this case no oxygen has to be supplied provided the range of surface area to volume is satisfied. Some kind of mechanical device would be necessary to ensure mixing. The degradation is best carried out at 35 C but is efficient at room temperature and there is still degradation at 15 C. Additives must be added to styphnic acid in order to obtain degradation. This can be done by adding corn steep liquor which satisfies the C/N ratio as well as the requirement for oligoelements and amino acids; tap water is

satisfactory. There is no need for an apparatus to control the pH since it is constant throughout the fermentation. Since the process is microaerophilic, cell synthesis is not as high as in an activated sludge system, thus reducing the amount of solids for disposal. The corn steep liquor could perhaps be replaced by domestic sewage, thus reducing the running cost of the system. There is no need for strict aseptic technique since the concentration of substrate used is toxic to many microorganisms. Wide variations in concentration of the effluent during the day may make it necessary to employ a dilution rate sufficiently lower than the maximum tolerated level to give an opportunity for the bacterial system to readjust to the fluctuating concentrations of substrate. Industrial wastes are often treated by close heterogeneous continuous systems of activated sludge, anaerobic digestion, and biological filtration but better results could be obtained by using homogenous continuous culture and could be an attractive process for the disposal of styphnic acid and similar compounds.

The degradation of styphnic acid in this study was obtained only under conditions of limited oxygen supply, unlike results obtained from other studies operated in highly aerated conditions.

The concentration of the substrate itself is important since nitrophenols can be bacteriostatic or bacteriocidal depending on the level used. The concentration of styphnic acid used in

this experiment was less than the dose of 1.5% reported by Cabridenc (1966) but a marked inhibition of degradation was found at a concentration above 0.015%. As reported by others the lower the concentration used the faster was the degradation rate.

The toxicity of nitrophenols is thought to be related to pH (Gundersen and Jensen, 1956). The increasing inhibitory effect of nitro-aromatic compounds with increasing acidity is apparently due to the greater permeability of the cytoplasmic membrane by undissociated molecules whose concentration depends on the hydrogen ion concentration. This explains why *Arthrobacter simplex* and this mixed culture are comparatively insensitive to the effect of picric acid and styphnic acid which are strong acids and are practically fully ionized over the pH range from 6.0 to 7.5.

Temperature has an effect on the degradation of styphnic acid. A wide range of temperatures from 15 C to 35 C was found at which there was a degradation of the substrate. Gundersen and Jensen (1956) and Cabridenc (1966) carried out their studies by incubating the organisms at 25 C while Jensen and Lautrup-Larsen (1967) used 25 C and 37 C. The wide range of temperature at which the substrate was degraded indicate that the same process could occur in soil or water over a good part of the year. Temperature affects growth of the organisms resulting in a faster degradation of the substrate as the numbers of organisms increase.

The pathway of styphnic acid degradation has never been investigated but generally nitro-compounds are detoxified, through the reduction of the NO_2 group or through the release of nitrite, before the attack on the benzene ring takes place. Cultures growing on styphnic acid did not release any ammonia and nitrite and no arylamine derivatives were detected on thin layer chromatography, using a solvent and an extraction technique similar to that used to detect arylamines derivatives from 2,4-dinitrophenol by Madhosingh (1961). The results are unlike those of Gundersen and Jensen (1956) who reported the release of large amounts of nitrite from cultures growing on p-nitrophenol, 2,4-dinitrophenol, dinitro-o-cresol, and picric acid and from Cartwright and Cain (1959) who observed the production of varying proportions of arylamines, ammonia, and nitrite from the aerobic metabolism of o-, m-, and p-nitrobenzoic acids.

Different compounds were tested to see if they could act as intermediates during the degradation of styphnic acid. These compounds represent the omission of one or several NO_2 groups from the styphnic acid molecule and include 2-nitroresorcinol, and 2,4-dinitrosoresorcinol. There was a rapid but incomplete attack on 2-nitroresorcinol and a slower and incomplete attack on 2,4-dinitrosoresorcinol. If these were intermediates a complete degradation of these compounds might be expected as they should be easily assimilated, also there would be a release of nitrite in the medium but

this was not found. Thus styphnic acid is probably not degraded through a stepwise release of the nitrite groups of the molecule. Indirect evidence of a ring cleavage of styphnic acid was obtained from the loss of absorbancy of the styphnic acid in the ultraviolet region as was also reported by Alexander and Lustigman (1966) for several aromatic compounds. However, the actual pathway of degradation is not known. A brown colour has been described to appear toward the end of degradation. Investigation showed no melanin pigments while Haider, Frederick, and Flaig (1965) reported reactions between amino acid compounds and phenols to produce humic acid. The brown substance was not soluble in 0.5 N NaOH; the first criteria of a humic compound. Microscopic examination showed that the brownish material appeared to be associated with the cells rather than diffused through the medium, but no material was released upon disruption of the cells by sonication. The brown substance was not characterized but several possible metabolites were ruled out. One possibility is that styphnic acid is reduced during the fermentation producing coloured compounds. Catalytic chemical reduction of styphnic acid gave a series of colour changes comparable with the biological degradation.

A study on the toxicity of the final product of the degradation of styphnic acid by the mixed culture should be undertaken to assess the relative toxicities of the substrate and product.

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