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Biodegradation of Resin Acid Sodium Salts

Abstract: The sodium salts of resin acids were readily degraded by microflora from two types of river water and from an activated sewage sludge. A lag phase with little or no resin acid salt degradation but rapid bacterial development occurred which was greatly extended by a decrease in incubation temperature. After this initial lag phase, the resin acid salts were rapidly decomposed with either of the three natural inocula. Sodium salts of levopimaric/palustric and dehydroabietic acids were most readily degraded, followed by abietic and neoabietic acid salts; pimaric and isopimaric acid salts were most resistant to biodegradation. Addition of 10% of neutralized spent bisulfite liquor did not affect cell growth or resin acid salt degradation but addition of acidic liquor restricted degradation by activated sludge until the pH had increased to 7.3.

Keywords: Resin acids · Resin acid sodium salts* · Waste water · Toxicity Bacteria · Biodegradation · Activated sludge · Microorganisms · Spent sulfite liquors

As early as 1931, Ebeling (*1*) recognized that resin acids were among the most harmful components of pulp mill waste water to fish, causing both death and contamination of the tissues. Hagman (*2*), in 1936, showed that resin acids at concentrations exceeding 1 ppm were toxic to fish. Later, in 1950, Van Horn, Anderson, and Katz (*3*) showed that sodium salts of resin and fatty acids were toxic to minnows at 1 and 5 ppm and to *Daphnia* at concentrations of 3 and 1 ppm, respectively. Mäenpää, Hynninen, and Tikka (*4*) found that resin acid salts were toxic to *Daphnia* at concentrations of 1 ppm and that the resin acids were toxic to *Daphnia* at concentrations exceeding 6 ppm. When the toxicity range of the resin acids and their salts is compared with that of the organic sulfides from kraft pulping operations (0.5 to 10.0 ppm) (*5–7*) or the chlorinated phenolic compounds suspected to be in bleach waste waters (0.25 to 3.0 ppm) (*8*), it is clear that the resin acids and their salts are potentially very hazardous compounds to aquatic animals.

A major source of resin acid salts in water is the waste from kraft pulping of resinous woods. Bergström (*9*) has estimated that a typical 40,000 ton/year kraft mill in Sweden discharges about 90 tons of resin each year when satisfactory scouring of waste water is practiced to minimize losses. The brown stock wash water from kraft pulping of pine wood in Finland has been shown to contain resin acid salts in concentrations as high as 100–400 ppm (*4*). Resin acids are also discharged with water when resinous woods are pulped by the sulfite processes. About half of the resin in *Pinus radiata* wood was removed with the spent bisulfite liquor, and additional quantities were removed with washing and screening waste water (*10*). Waste waters containing toxic concentrations of resin acid salts are not restricted to effluents from chemical pulping operations. Large quantities of resin acids are used by the paper industry as an additive and a proportion is discharged with paper mill waste water (*4*). Row and Cook (*11*) have reported that some waste waters from debarking, groundwood pulping, and fiberboard production are toxic to fish because of high concentrations of resin acids.

We have found no published literature concerned with parameters important to the biological decomposition of resin acids or their salts. Therefore, we have conducted a series of experiments to define the importance of the source of microorganisms, temperature, presence of spent bisulfite liquor, and pH on the biodegradation of resin acid salts and have briefly examined the extent of degradation of these compounds.

RESULTS AND DISCUSSION

Preliminary Screening of Bacterial Isolates

A total of 69 bacteria, isolated from wood sources, were screened for their tolerance and ability to utilize the sodium salts of palmitic, oleic, and a mixture of resin acids.[1] Of the wood-inhabiting bacteria screened, 54% were completely inhibited by the highest concentration (ca. 40 ppm) of fatty and resin acid salts in gradient plates. Few of the bacteria tested appeared to utilize the sodium salts of palmitic, oleic, or the mixture of resin acids as their major carbon source.

The bacteria which showed some potential for utilizing resin acid salts were: *Bacillus lentus; Bacillus pantothenticus; Bacillus pulvifaciens; Bacillus pumilus; Bacillus polymyxa; Bacillus subtilis; Escherichia coli; Flavobacterium* sp.; *Pseudomonas* sp.; Unidentified G-187; the Unidentified G-195. Solutions of the resin acid salt mixtures at a concentration of 40 ppm in Dubos buffer (pH 7.4) were inoculated with the previously mentioned bacterial isolates and incubated at 27°C for periods up to 6 days while resin acid salt concentration and cell growth were measured periodically. There was no significant change in resin acid salt concentration with any of these bacteria. Examination of cell counts gave inconclusive results.

It is well known that isolated pure

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[1] A compilation of the bacterial isolates screened and their response to addition of fatty and resin acid salts can be obtained from H. Greaves.

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culture systems are often unable to degrade compounds, while the same microorganisms in mixed culture systems may rapidly decompose them (*12, 13*). Therefore, the inability of these isolates to degrade resin acid salts does not prove that of the 69 bacteria screened, none may be involved in the degradation of these compounds when the bacteria occur in mixed natural microflora. Raynaud and co-workers (*14*) have isolated two bacterial species, capable of utilizing rosin as the sole carbon source, which were named *Flavobacterium resinovorum* and *Pseudomonas resinovorans*. Both a *Flavobacterium* and a *Pseudomonas* sp. examined in our studies appeared to utilize the resin acid salts when examined on agar plates, but this could not be verified in liquid culture studies.

Microbial Activity of River Waters and an Activated Sludge

Solutions containing 40 ppm of resin acid salts in Dubos buffer (pH 7.4) were inoculated with samples from the Yarra River at South Melbourne, Victoria, from the North Pine River at Petri, Queensland, and from an activated sludge at the Heatherton sewage works in Victoria. When the resin acid salts were incubated at 27°C with the activated sludge or Yarra River inoculum, all resin acid salts were decomposed to concentrations below measurable levels within 12 to 15 hr, after an initial lag phase of 18 to 20 hr during which little or no change in resin acid salt concentration occurred (Fig. 1). Inoculation with the North Pine River sample required about 28 hr before degradation of resin acid salts occurred, and they were degraded to concentrations below measurable levels after a total of 40 hr at 27°C. There was little difference in the rate of degradation between the three sources of microorganisms after the decomposition became appreciable. Differences in degradation rates of individual resin acid salts were common to all sources of microorganisms; those resin acid salts present in greatest proportions, levopimaric/palustric[2] and dehydroabietic, were decomposed more rapidly than abietic and neoabietic acid salts; pimaric and isopimaric acid sodium salts were the most resistant to degradation. When incubated with North Pine River water, neoabietic acid was much more resistant to degradation than was abietic, and its stability was similar to that of pimaric acid.

Bacterial cell populations of the three microbial sources developed at similar rates, the total viable cell count increasing by about 10^5 times within 20 hr of incubation at 27°C (Fig. 2). During this time there was no significant change in the concentration of resin acid salts. When marked degradation occurred, the bacterial population increased a further 10^8 times. Total cell development during the full incubation period with 40 ppm of resin acid salts was about 10^{12}–10^{13} times (Fig. 2).

To examine the degradation rate of resin acid salts by these microorganisms under conditions similar to their natural environment, solutions were incubated at 12°C (Fig. 1). The lag phase when there was little or no degradation of resin acid salts increased to 80 hr with activated sludge, to 100 hr with the Yarra River, and to 140 hr with the North Pine River inoculum. Lowering the temperature had more effect on this initial period of cell growth than on the subsequent degradation rate when decomposition of the resin acid salts became appreciable. After degradation began, the incubation period required to degrade the resin acid salts below measurable concentrations increased to 20 hr with activated sludge, to 25 hr with Yarra River, and to about 30 hr with North Pine River inocula. The differences in degradation rates of individual resin acid salts which were common to the three sources of microorganisms were more evident at this lower incubation temperature. There was considerable degradation of the levopimaric/palustric and dehydroabietic acid salts before there was much degradation of abietic and neoabietic acid salts. Pimaric and isopimaric acid salts were very resistant to microbial attack; on several occasions traces of these latter compounds were found after 164 hr of incubation at 12°C.

While there was a long period of little or no resin acid salt degradation at 12°C, there was considerable bacterial development after 16 hr of incubation and the viable cell-count increased by about 10^9 times before there was appreciable resin acid salt degradation (Fig. 2). The viable bacterial cell population at the point when resin acid salt degradation became significant was about 10^4 times greater at 12° than at 27°C.

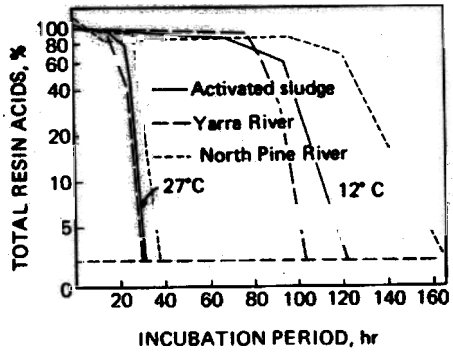

Fig. 1. Effect of temperature and source of microflora on rate of resin acid salt degradation.

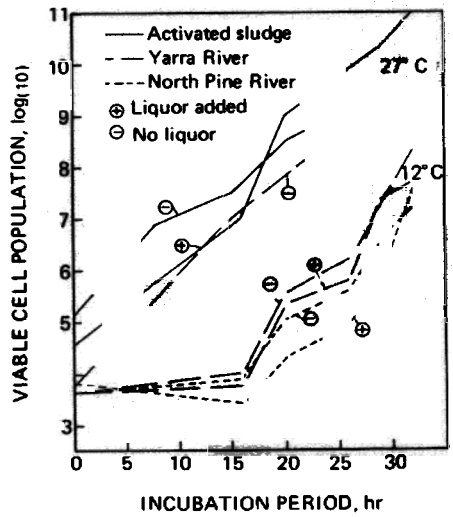

Fig. 2. Effect of temperature and source of microflora and neutralized bisulfite spent liquor on rate of cell development.

Effect of Spent Bisulfite Liquor

It was of interest to know what effect the presence of a spent liquor from bisulfite pulping of *Pinus radiata* wood might have on the rate of resin acid salt degradation with the three sources of microorganisms. The spent liquor was adjusted to pH 7.1 with NaOH and added to the solution of resin acid salts in Dubos buffer at a concentration corresponding to 10% of the original liquor.

Addition of the neutralized liquor caused marked alterations in the composition of the resin acids. However, substantial quantities of recognizable resin acids remained. Compounds measured in these cultures containing spent liquor were: dehydroabietic, abietic, pimaric, and material with the relative retention time of levopimaric/palustric acid methyl ester which was probably methyl palustrate. Addition of the equivalent of 10% of neutralized bisulfite spent liquor had no significant effect on the rate of resin acid salt degradation when incubated with the activated sludge sample at either 12° or 27°C or with the microorganisms from either of the river waters when incubated at 12°C (Fig. 3). The neutralized spent liquor had no influence on the relative rates of decomposition of individual resin acid salts; dehydroabietic acid salt was degraded most rapidly, while pimaric acid salt was the most resistant.

[2] Levopimaric acid and palustric acid methyl esters were not separated using DEGS columns so the sum peak area was measured and reported as levopimaric/plaustric acids.

The initial rate of bacterial cell development was not influenced by the presence of neutralized spent liquor (pH 7.1), but bacterial growth increased beyond measurable levels when resin acid salt degradation became significant (Fig. 2). During the period when rapid resin acid salt degradation occurred, there was a darkening of the color of the culture liquor and the pH increased from 7.1 to 8.1.

While there was no effect on culture systems when 10% of neutralized spent liquor was added (pH 7.1), the addition of the acidic liquor to the resin acid salts in Dubos buffer (pH 4.7) prevented any significant change in resin acid salt concentration after 50 hr of incubation at 27°C with the activated sludge sample. The viable cell count did not increase when the pH of the culture liquid was so low.

Influence of pH

To gain more information on the importance of pH on the degradation rate of resin acids in the presence of spent bisulfite liquor, the liquor was back-titrated with NaOH and added to the resin acid salts in Dubos buffer at concentration equivalent to 10% of the original liquor with initial pH levels of 5.5, 6.0, and 6.5. During the course of incubation with the activated sludge at 27°C, the solution became increasingly dark and the pH increased to 7.5 to 8.1, depending upon the amount of NaOH used to back-titrate the spent liquor (Fig. 4).

There was no significant change in the concentration of resin acid salts while the pH was below 7.2. However, after the pH had reached about 7.3, there was a rapid degradation of the resin acid salts in cultures at all three initial pH levels (Fig. 4). The effect of initial pH on the degradation rate of resin acid salts was associated almost entirely with the time required for the pH to increase to 7.3. Neither the rate of total resin acid salt degradation nor the relative rates of degradation of individual resin acid salts was influenced much by the initial pH of the solution. It would appear that microbial attack on lignosulfonic acids present in the spent liquor is a most important factor controlling biodegradation of resin acid salts in solutions containing spent bisulfite liquors at mildly acidic pH conditions.

Extent of Resin Acid Salt Degradation

To obtain a concept of the extent of resin acid salt degradation with microflora from natural waters, ^{14}C labeled resin acid salts were incubated at 27°C with the North Pine River sample. The amount of CO_2 liberated, as well as radioactivity of the $BaCO_3$ recovered, was measured at intervals throughout the incubation period. A control incubation of unlabeled resin acid salts was used to obtain information on the rate of resin acid salt degradation and bacterial cell development. Results are summarized in Fig. 5.

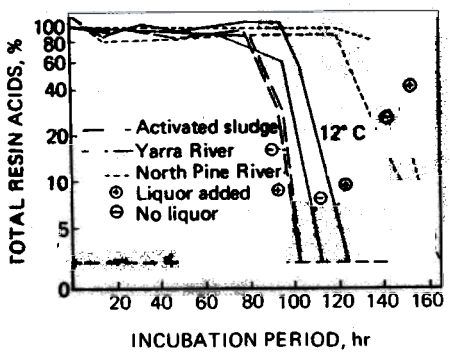

Fig. 3. Effect of neutralized spent bisulfite liquor on rate of resin acid salt degradation.

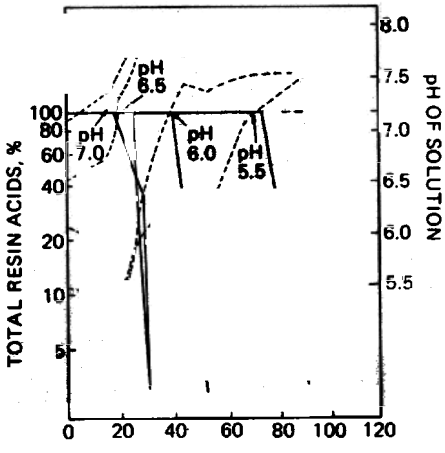

Fig. 4. Effect of initial pH on the rate of resin acid salt degradation.

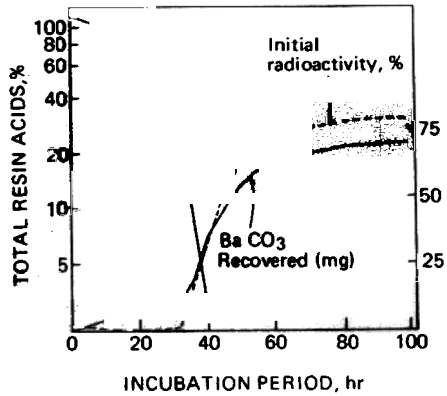

Fig. 5. Extent of ^{14}C labeled resin acid salt degradation, North Pine River water-27°, pH 7.5, no spent bisulfite liquor.

There was substantial bacterial development during the initial 30 hr when no significant change in resin acid salt concentration occurred. Large quantities of CO_2 were liberated during this period, but the $BaCO_3$ recovered was not radioactive. The absence of radioactivity in the CO_2 liberated during this initial phase lends support to the thesis that bacterial growth in this stage of incubation is supported by nutrients in the inoculum. Contaminants in the resin acid salt substrate such as small amounts of fatty acids or their esters would be radioactive.

Degradation of the resin acid salts was appreciable between 28 and 40 hr of incubation, and there was a marked increase in the amount of CO_2 liberated during the incubation period from 30 to 50 hr. The $BaCO_3$ recovered during this period was radioactive. When the cumulative amount of radioactivity recovered as $BaCO_3$ is compared with the amount of radioactivity added with the resin acid salts, it is apparent that the resin acid salts were rapidly metabolized to CO_2 as about 80% of the added radioactivity was recovered as CO_2 after 90 hr of incubation.

EXPERIMENTAL

Resin Acids

Resin acids were obtained by precipitation of their cyclohexylamine salts from acetone extracts of the wood of *Pinus pinaster* and wood of a *Pinus radiata* tree which had been grown for a period in $^{14}CO_2$ atmosphere (*15*). To insure removal of all of the amine, the resin acids were washed thoroughly with a large excess of boric acid. Sodium resinates were salted from solutions of the resin acids in a slight excess of warm sodium hydroxide.

Screening of Bacteria

A total of 69 wood-inhabiting bacteria were screened for their ability to utilize fatty or resin acid sodium salts using diffusion gradient plates prepared with a Dubos mineral salt solution agar as the basal medium (*16*). The second wedge contained in addition to mineral salts and agar, filter sterilized solutions of the sodium salts of palmitic, oleic, or the mixture of resin acids obtained above. Plates were incubated at 25°C for 24 hr except where thermophilic microorganisms were involved in which case the temperature was 45°C. In addition, the same screening experiments as mentioned previously were made, except that the agar medium also contained the following nutrients: peptone, 1.0%; casamino acids, 0.5%; and glucose, 0.5%.

Liquid shake cultures were made using 500-ml conical flasks, each containing the basal medium of Dubos salt buffer, 5 ml of inoculum, 49 ppm of resin acid salts, and in some instances, the equivalent of 10% by volume of spent bisulfite liquor which had been adjusted to various pH levels. Control incubations were prepared using autoclaved inoculum. After various incubation periods, samples were withdrawn aseptically from the cultures and the bacteria removed by centrifugation. The supernatant was pipetted off, the cells washed with Dubos buffer, and the bacteria centrifuged down a second time. The pH of the first supernatant was determined, the two supernatants combined, and then stored at 3°C until analyzed for resin acid salt content.

The supernatant liquors were treated with a saturated boric acid solution, the resin acids extracted into diethyl ether, methylated with etheral diazomethane, and the methyl esters diluted to 3 or 5 ml with acetone. Duplicate analyses were made of these solutions of resin acid methyl esters by gas–liquid chromatography using conditions described previously (*17*). Viable cell populations in 1 ml aliquots were estimated using a series of 10-fold dilution plates. After incubation at 25°C for 24 hr, colonies were counted on plates showing between 30 and 300 colonies.

Radioactive Experiments

To determine the extent of degradation of the resin acid salts, radioactive resin acid salts were dissolved in Dubos buffer at 50 ppm and the solution inoculated with the North Pine River sample. Carbon dioxide free air was passed through the incubation flask, two $Ba(OH)_2$ traps, and through a flow meter at 10 ml/min. The $BaCO_3$ was collected at intervals, weighed, and its radioactivity determined with a geiger tube. A parallel incubation with the same inoculum was made to determine the rate of cell development and resin acid salt degradation.

LITERATURE CITED

1. Ebeling, G., *Von Wasser* 5 (11): 192 (1931).
2. Hagman, N., *Finnish Paper Timber J.* 18 (1): 32 (1936).
3. Van Horn, W. M., Anderson, J. B., and Katz, M., *Tappi* 33 (5): 209 (1950).
4. Mäenpää, R., Hynninen, P., and Tikka, J., *Paperi ja Puu* 4(a): 145 (1968).
5. Haydu, E. P., Amber, H. R., and Dimick, R. E., *Tappi* 35 (12): 545 (1952).
6. Seppovaara, D. and Hynninen, P., *Paperi ja Puu* 6 (1): 11 (1970).
7. Cole, A. E., *J. Pharm. Exp. Therap.* 54 (4): 448 (1935).
8. Servisi, J. A., Werden, R. H., and Martens, D. W., International Pacific Salmon Fisheries Commission, New Westminster, B. C., Progress Report 17 (1969).
9. Bergström, H., *Svensk Papperstid.* 56 (3): 75 (1953).
10. Nelson, P. J. and Hemingway, R. W., *Tappi* 54 (6): 468 (1971).
11. Row, R. and Cook, R. H., *Pulp Paper Mag. Can.* 72 (6): 38 (1971).
12. Gottlieb, S. and Pelczar, M. J., *Bact. Rev.* 15: 55 (1951).
13. Kleinert, J. M. and Joyce, C. S., *Svensk Papperstid.* 62: 37 (1959).
14. Raynaud, M., Daste, P., Grossin, F., Biellmann, J. F., Wenning, R., *Ann. Inst. Pasteur* 115 (104): 731 (1968).
15. Shain, L. and Hillis, W. E., unpublished work.
16. Dubos, R. J., *J. Bact.* 15 (4): 223 (1928).
17. Hemingway, R. W., Nelson, P. J., and Hillis, W. E., *Tappi* 54 (1): 95 (1971).

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