Microbial Utilization of Pinus radiata Bark

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A screening program using suspensions of ground bark in mineral salts media, or extracts prepared from ground bark by treating with hot water, sulfuric acid, ammonium hydroxide, or sodium hydroxide, yielded more than 200 pure cultures of fungi, yeasts, and bacteria. Only 38 of these gave good growth on liquid bark media. All were filamentous fungi, although many bacteria and yeasts were among the cultures that failed to give appreciable growth. Species of Penicillium, Scopulariopsis, Aspergillus, Trichoderma, Cladosporium, and Fusarium were among the most actively growing cultures. Cell biomass yields, as measured by cell nitrogen determinations, were too low for economic production of single cell protein.

The use of bark as a fermentation substrate has been totally neglected until the past 2 years. Eriksson (5) mentions in a review paper on microbial decomposition of bark that sugars for fermentation were produced by adding cellulase and hemicellulases to bark to break the polysaccharides down to monosaccharides. Another approach was that of Nordstrom (12), who cultivated Aspergillus fumigatus on bark and extracts of bark. Bailey and Pickmere (3) reported on the use of cellulolytic fungi to convert polysaccharides in Pinus radiata bark into sugars, which could be used as stockfood or as a fermentation substrate.

None of these publications has come to grips with the question of whether an economically successful industrial fermentation could be developed with bark as the substrate. The present work addresses itself to this question with respect to the bark of P. radiata, the principal species used in forestry in New Zealand (6).

MATERIALS AND METHODS

Preparation of bark and bark extracts. P. radiata bark, from the mechanical debarker at Bajent's sawmill, Wakefield, New Zealand, was air-dried to constant weight, brushed to remove adherent dirt, and ground to less than 1-mm particle size in a Wiley mill.

A wide range of extraction and chemical treatment procedures was carried out to solubilize as much of the bark as possible, including exhaustive sequential Soxhlet extraction with hexane, ether, benzene, ethanol, methanol, and water, boiling gently on a hot plate, and, in the case of aqueous solvents only, autoclaving for 30 min at 120°C at a solvent-to-bark ratio of 10:1 (vol/wt). Some extracts were made by blending for 2 min in a Waring blender with hot or cold water or 1% H₂SO₄, followed by a similar treatment after allowing the mixture to stand for 30 min. The mixtures were then centrifuged or filtered through Whatman no. 1 paper. In some cases, the solvents were evaporated in vacuo on a rotary evaporator and the residue was weighed to determine yield. In other cases, suitable analytical methods were applied.

Analytical procedures. Qualitative and semiquantitative determinations of the carbohydrates present in bark extracts were carried out by descending paper chromatography of the extracts and of their acid hydrolysates in the solvent system ethyl acetate-pyridine-water (8:2:1, vol/vol/vol), detecting sugars by the methods of Saini (14) and Trevelyan et al. (16). A visual comparison of spot intensity with a range of standards on the same chromatogram allowed surprisingly accurate estimations of sugars to be made. Total tannins and chemical oxygen demand were determined as described in Standard Methods (2). Total cell biomass of microorganisms was determined as dry weight of washed mycelia after centrifugation or after filtration through a 40-mm Sartorius glass-fiber filter. Cell nitrogen was determined by the micro-Kjeldahl method (7). Cellulose analyses were carried out by a semi-micro anthrone method (17). Klason lignin was determined gravimetrically essentially as described by Adams (1), with the additional step of removing "phenolic acids" (phenolic polymers related to condensed tannins) with 1% NaOH at 90°C prior to the total acid hydrolysis of carbohydrate (9). The total weight loss, which occurred as a result of these two treatments, was considered to represent the combined weight of holocellulose and phenolic acids. An approximate figure for the content of phenolic acids was obtained by difference after a separate determination of holocellulose (15).

Microbiological methods. Many isolation experiments were carried out on an enrichment medium composed of a 10% suspension of ground bark in water supplemented with 1% NH₄H₂PO₄. The bark contained adequate amounts of the mineral nutrients Mg, Ca, K, Na, S, Mn, Fe, Zn, and Co, as shown by chemical analysis. Alternatively, bark ex-
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needles, soil, compost, sawdust, and rotting leaves. The enrichment cultures were incubated at 28°C on a gyratory shaker with a 1-cm stroke at 200 to 250 rpm for 5 to 10 days. When microbial growth had occurred, as determined by microscopic examinations, the cultures were streaked out on either potato-dextrose agar (BBL) or bark extract agar prepared by adding agar (2%) and NH4H2PO4 (0.1%) to the filtrate of a 10% suspension of ground bark, which had been autoclaved in distilled water for 30 min at 120°C.

Stock cultures were maintained on bark extract agar. For the initial screening experiments, a 10-ml portion of sterile bark medium in a 100-ml Erlenmeyer flask was inoculated with one loopful of growth from a fresh agar slant culture. For quantitative growth studies on fungi, shake flasks were inoculated with 105 to 106 conidia from a spore suspension in 0.01% Tween 80 in 1% NH4H2PO4.

Laboratory fermentor studies. Scale-up studies of cell biomass production were carried out at 28°C in two 7.5-liter fermentors (New Brunswick Co.) charged with from 3 to 5 liters of medium. Each fermentor, with medium and associated tubing, was autoclaved at 120°C for 30 min, cooled, and then inoculated with at least 106 conidia of the test fungus grown on bark agar plates. Alternatively, 100 ml of a heavy suspension of chopped mycelium was used as an inoculum. The cultures were aerated with sterile air at from 1 to 6 liters/min, and the stirring rate was 150 to 250 rpm. Each day a sample of from 10 to 50 ml was removed and filtered, and the residual chemical oxygen demand of the filtrate was determined. In some runs it was necessary to add sterile silicone Antifoam (silicone MS Antifoam emulsion B; Hopkin and Williams, Ltd., Essex, England).

RESULTS

Composition of P. radiata bark and bark extracts. The overall composition of P. radiata bark is presented in Table 1. Since fermentations of insoluble substrates rarely proceed at rates sufficiently high for economically feasible industrial fermentations (10), we have confined ourselves largely to the use of different soluble extracts of bark as substrates in our isolation and fermentation studies. Preliminary results indicated that, although alkaline extraction dissolved up to 70% of the bark by weight, media prepared with such extracts invariably supported only very poor microbial growth. The most promising results were obtained with water and dilute sulfuric acid extracts, and analytical data on these are given in Table 2.

Isolation of microorganisms. The enrichment procedure described in Materials and Methods yielded more than 200 pure cultures, mostly fungi, which were subsequently screened for cell biomass production on bark extracts supplemented with 1% NH4H2PO4. None of the bacteria or yeasts gave good yields, but 38 of the fungal cultures were selected on this basis for further investigation. Most of these were identified to the genus level by cultural and microscopic examinations. Protein yields were determined for these cultures on both water and sulfuric acid extracts of bark supplemented with 1% NH4H2PO4 at pH 4.1, 6.3, and 7.0. The best yields were produced by a

**Table 1. Overall chemical composition of P. radiata bark**

| Constituent                        | % Dry wt |
|-----------------------------------|----------|
| Lipids (by sequential Soxhlet extraction with hexane and benzene) | 6        |
| Flavonoids and condensed tannins (by ethanol extraction of the residue from above) | 21       |
| Holocellulose (containing 12% cellulose) | 26       |
| Klasson lignin                     | 11       |
| Phenolic acids                     | 39       |
| Free sugars (by paper chromatography of hot-water extracts) | 1        |
| Ash                               | 3        |

**Table 2. Composition of extracts prepared by autoclaving bark with water (W) and 1% sulfuric acid (S)**

| Composition | Extract W | Extract S |
|-------------|-----------|-----------|
| Chemical oxygen demand | 22 | 24 |
| Total solids | 23 | 29 |
| Tanninsa | 15.6 | 3.5 |
| Carbohydrates | | |
| Glucose | 0.5 | 1.0 |
| Galactose | Trace | 0.6 |
| Mannose | Nil | 0.2 |
| Fructose | 0.3 | 0.2 |
| Arabinose | 0.2 | 1.5 |
| Xylose | Nil | 0.6 |
| Sucrose | 0.1 | Nil |
| Uronic acids | Nil | Traces |
| Polysaccharidesb | 0.1 | Traces |

a Determined colorimetrically using purified tannins from P. radiata bark as the standard. The low value for extract S probably reflects the alteration of tannin structure, and hence of extinction coefficient, on heating with sulfuric acid.
b Estimated by paper chromatography of acid hydrolysates.

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strain of *Penicillium* sp. (2 to 3 mg/g of bark on water extract and 10 to 12 mg/g of bark on sulfuric acid extract at pH 4.1 and 6.3) and by a strain of *Scopulariopsis* sp. (8 to 9 mg/g on water extract and 12 to 13 mg/g on sulfuric acid extract at pH 7.0). Six cultures, including F11 and F23, were identified as this *Penicillium* strain, which gave abundant green conidia on bark agar, Sabouraud's agar, or Czapek's agar. Two cultures of the *Scopulariopsis* strain were isolated. Other strains of *Penicillium* sp., *Aspergillus niger*, *Cladosporium* sp., *Fusarium* sp., *Trichoderma* sp., *Sporiopsis* sp., and *Stemphylium* sp. gave lower yields. Cultures of *Candida utilis* and the wood-rotting fungi *Peniophora sacra*, *Trametes versicolor*, *Polyergus biennis*, and *Trametes* sp. gave negligible yields.

**Polyphenol oxidases and tannin polymerization.** During the early screening experiments it was noticed that many of the bark extracts slowly produced a dark brown precipitate of polymerized tannins over the period of incubation. It was later discovered that many of the fungal isolates produced extracellular polyphenol oxidases, which greatly accelerated the formation of these insoluble polymerized tannins (8), and since most of this precipitated material adhered strongly to the fungal mycelium conventional gravimetric analysis was impossible.

**Effect of added nutrients on growth rate.** A series of experiments was set up employing the shake flask technique and gravimetric determinations of total cell biomass, as well as chemical oxygen demand determinations, to investigate the effects of added nitrogen, phosphorus, molybdenum and other trace elements (13), and yeast extract on the growth rates of selected cultures. Supplementation with added phosphate, molybdenum, or trace elements had little or no effect on the growth rates of most of the fungi tested, 0.1% yeast extract had either no effect or a slight stimulatory effect, and nitrogen in the form of 1 to 2% NH₄H₂PO₄ or (NH₄)₂SO₄ had a highly stimulatory effect.

**Laboratory scale-up phase.** The laboratory scale-up phase of the project was carried out with two media, based on the water and sulfuric acid extracts of bark described earlier. Samples were removed every day for chemical oxygen demand measurements, but daily cell biomass determinations could not be made, since large masses of mycelia adhered to the stainless steel components of the fermentor during the run. The final fermentor contents were harvested by filtration. The wet residues were weighed and well mixed, and samples were removed for analytical purposes. The data for three such dual runs with isolates F23, B4, and F11 are shown in Table 3. It is apparent that insoluble polymerized tannin accounted for 82 to 96% of the apparent mycelial yield after growth on the water extract and for 50 to 65% after growth on the sulfuric acid extract.

**Biodegradation of whole bark.** To evaluate the rate of microbial degradation of whole *P. radiata* bark by our fungi, weighed samples of bark were autoclaved with solutions of seven different mineral salts media chosen to explore the variables of different nitrogen sources and trace element requirements. After inoculation with soil, sawdust, decaying bark, or conidia from the pure cultures, the cultures were incubated under static conditions for 28 days and then dried and weighed. In agreement with the results reported by Swedish workers (5), less than 5% of the bark weight was lost in these experiments, and protein yields were low, from 0 to 9.4 mg/g of bark. Acid treatment of whole bark did not improve protein yields.

**DISCUSSION**

As estimated by gravimetric methods, the maximum yields of "cell biomass" material produced by fungal growth on extracts of bark ranged from 5 to 18% of the dry weight of the bark extracted. Chemical analyses of the "cell biomass" material showed, however, that from 50 to 96% of it consisted of condensed tannins. Thus the true cell biomass yields were less than 5% of the bark weight in nearly all cases. Cell nitrogen determinations confirmed the gravimetric determinations in that the protein yields obtained could be accounted for if the sugars alone in the bark or bark extract (1 to 4% of the bark's weight) were utilized as a substrate. Furthermore, chemical analyses confirmed that our most active fungus cultures did indeed consume all of the sugars in 4 or 5 days of incubation.

No evidence for actual utilization of tannins by the fungi was ever obtained, which is in agreement with findings by Benoit et al. (4) during their studies on the effects of wattle tannin on microbial degradation of organic compounds in soil. Similarly, Nordstrom (12) found that *Aspergillus fumigatus* grown on ground Norway spruce bark preferentially utilized the carbohydrates; lignin was not degraded.

Applying a calculation method similar to Nordstrom's to our data on cell nitrogen, we derive protein values of 9 to 13 mg/g of bark from our best fungi. Assuming that the cell material is 33% protein, a good average value for filamentous fungi, this is equivalent to 27 to 39 mg of cell material per g of bark, or 2.7 to 3.9% of the bark's weight. Thus our yields from
### Table 3. Batch fermentation of bark extracts with filamentous fungi

| Determination | Penicillium sp. (F11) | Cladosporium sp. (F12) | Penicillium sp. (F11) |
|---------------|-----------------------|------------------------|-----------------------|
|               | Run 1A                | Run 1B                 | Run 2A                | Run 2B                 | Run 3A | Run 3B |
| Incubation time (days) | 3                    | 3                      | 6                    | 6                      | 6      | 6      |
| Medium (W, water; S, sulfuric acid)* | W                    | S                      | W                    | S                      | W      | S      |
| Total volume (ml) | 5,000                 | 5,000                  | 3,000                | 3,000                  | 4,000  | 4,000  |
| Initial pH | 4.1                    | 4.1                    | 4.1                  | 4.1                    | 4.1    | 4.1    |
| Final pH | 3.5                    | 3.4                    | 3.8                  | 3.8                    | 3.5    | 3.4    |
| Initial substrate (g) | 94.0                  | 57.0                  | 39.0                 | 28.8                   | 77.2   | 47.2   |
| Remaining substrate (g) | 7.7                    | 6.6                    | 30.0                 | 14.6                   | 5.7    | 9.0    |
| Substrate apparently consumed (difference) | 86.3                  | 50.4                  | 9.0                  | 14.2                   | 71.5   | 38.2   |
| Solid residue (g) (mycelium plus polymerized tannins) | 45.1                  | 23.2                  | 7.9                  | 8.1                    | 40.5   | 15.0   |
| Cell biomass after cold 0.5 N NaOH extraction (g) | 8.1                    | 11.6                  | 0.3                  | 3.0                    | 1.6    | 5.3    |
| Polymerized tannins as % of solid residue | 82                    | 50                    | 96                   | 63                    | 96     | 65     |
| Yield [(corrected biomass)/(initial substrate)] (%) | 8.6                    | 20.4                  | 0.8                  | 10.4                  | 2.1    | 11.2   |
| Protein yield (mg/g of initial bark) | 2.2                    | 6.9                    |                      |                        |        |        |

* Medium W was supplemented with 6.3% (NH₄)₂SO₄; medium S was supplemented with 6.6% of concentrated NH₄OH.

P. radiata bark are considerably lower than Nordstrom's yields from Norway spruce bark. This is only of academic interest, however, as both our production rates and Nordstrom's are far below those of commercially feasible single cell protein fermentations. Our best rate of 39 mg/g of bark per 100 h is equivalent to 0.04 g/liter per h. Nordstrom's rate was 0.1 g/liter per h. Compare these rates with Mellor's figure of 3.7 g/liter per h for Candida utilis growing on glucose (11).

A considerable effort was expended in attempts to develop a method of separating the interfering tannins from fermentable sugars in the hope that it could be scaled up to an economically feasible commercial process. The methods tried included liquid-liquid solvent extraction, chromatography on columns of activated carbon, silica gel, calcium carbonate, and Sephadex, precipitation of tannins with lead acetate, ferric chloride, lime, magnesium, and gelatin, and ultrafiltration with Bio-Rad cellulose acetate microtubules. In no case was a clean separation obtainable without either large amounts of expensive reagents or laborious multistep treatments. At present we can see no way in which an economically useful substrate for industrial fermentations can be prepared from P. radiata bark.

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