CHARACTERIZATION AND ENUMERATION OF THE RESIN ACID-DEGRADING BACTERIAL POPULATION OF A SEQUENCING BATCH REACTOR: AN EMPHASIS ON THE ISOPIMARIC ACID-DEGRADING BACTERIA

by

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ABSTRACT

Resin acids are naturally occurring compounds produced by conifers and released by pulp and paper mills. These compounds are problematic to papermaking processes and are highly toxic to aquatic organisms. Since basic microbial research on biodegradation of resin acids is limited, it was of interest to investigate the resin acid-degrading community of an established sequencing batch reactor operated at temperatures between 20° and 50°C; to determine if operating temperature changes affected the species present. Specificities for resin acids and other substrates, temperature ranges for and induction of resin acid degradation were characterized for several isolates.

Five resin acid-degrading bacterial strains were isolated from the sequencing batch reactor at either 20° or 40°C. AbA-1 grew on the abietanes, dehydroabietic and abietic acids. IpA-1, IpA-2, IpA-13, and AbA-5 grew on the pimaranes, isopimaric and pimaric acids, and the above abietanes. These latter four strains are the first reported isolates which grow on the pimaranes as a sole organic carbon and energy sources. Carbon mass balance determinations for IpA-1, IpA-2 and IpA-13 could not conclusively prove that isopimaric acid was completely mineralized because one-third of the isopimaric acid carbon remained as dissolved organic carbon; however, partial mineralization was shown. IpA-1 did not produce a biosurfactant during growth on isopimaric acid. Induction studies confirmed removal of resin acids by isopimaric acid degraders requires induction by growth on IpA.

When the five strains were compared biochemically, the results suggested that increasing the temperature of the sequencing batch reactor from 20° to 40°C changed the resin acid-degrading bacterial community, from one with at least two gram-negative populations, abietane and pimarane-degrading ones, to a less diverse one with a gram-positive population having the ability to remove both abietanes and pimaranes. Most probable number determinations showed

increasing the reactor's temperature above 40°C decreased the culturable isopimaric, dehydroabietic, and abietic acid-degrading populations to 10^3 per 100 ml. The remaining populations above 40°C required additional energy sources to remove resin acids. The resin acid-degrading community was resilient to rapid changes in resin acid concentration up to 3000 μM and pH shifts to 6 or 8.

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ABBREVIATIONS AND SYMBOLS

AbA	abietic acid
C	celsius
Cl-DhA	12/14-chlorodehydroabietic acid
12-Cl-DhA	12-chlorodehydroabietic acid
14-Cl-DhA	14-chlorodehydroabietic acid
3-CB	3-chlorobenzoate
cm	centimeter
CTMP	chemi-thermomechanical pulp
DhA	dehydroabietic acid
DOC	dissolved organic carbon
FID	flame ionization detector
g	gram
GC	gas chromotograph
h	hour
HPLC	high performance liquid chromatography
IpA	isopimaric acid
kD	kilodaltons
l	liter
LC_{50}	lethal concentration killing 50%
m	meter
MES	2-[N-Morpholino]ethanesulfonic acid
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MPN	most probable numbers
nm	nanometer
OD	optical density
PCP	pentachlorophenol
PiA	pimaric acid
ppm	parts per million
rpm	revolutions per minute
SBR	sequencing batch reactor
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	seconds
σ_{20}	dynamic surface tension at a bubble age of 20 sec
t_x	initial time, in seconds, for surface tension change
TMP	thermomechanical pulp
µg	microgram
µl	microliter
µm	micrometer
µmol	micromoles
µM	micromolar

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I would like to thank, Bill Mohn for his support in terms of guidance, encouragement, and patience, and for providing me an example of what a scientist is, without this I would not have developed into the scientist I am now. I would also like to thank my present and former lab colleagues Vince Martin, Annette Muttray, Tai Man Louie, Karolina Westerberg, and Roshie Banisadr for scientific discussion, advice, support, and good conversation. A special thanks goes to my proof-readers for their patience and perseverance through my endlessly long sentences, to Linda Sandercock and Anne Greene for their friendship, and to my family for their love and emotional support throughout this degree.

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DEDICATION

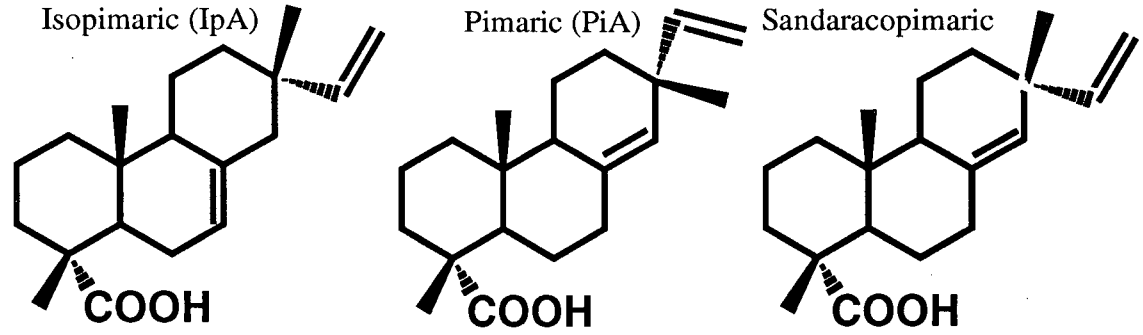
I would like to dedicate this thesis to my husband, Mark, who encouraged me to take the first step on the long path to the completion of this Master's thesis, for his continual support throughout this process, and for his unconditional love that made and still makes all things possible.

INTRODUCTION

Resin acids are naturally occurring, tricyclic, diterpenoid compounds, which are produced by many tree species, particularly conifers. They can be divided into two families, the abietanes and the pimaranes, based on the C-13 substituent (Fig. 1). The abietane family has an isopropyl side chain at the C-13 position, while the pimarane family has a methyl and a vinyl constituent at this position. Resin acids, along with fatty acids, are included within the "extractives" component of wood, that makes up less than 8% by weight of the total wood. A review by Swan [47] summarized data which indicated these resin acids are located in the resin canals and the ray parenchyma cells of many conifers, which are known as softwoods. It is important to understand that this resin acid content not only varies between different tree species, but it also varies within a given species and within the same tree. Wallin and Condren [51] showed that pine species contain the greatest percentage of resin acids, 1.5% by weight of oven dried wood, while other softwoods only contain roughly 0.1%. In contrast, most hardwood species contain negligible amounts of resin acids. Within the same tree species, Swan's review indicated that larger diameter, older trees possess a greater percentage of resin acids. Also, the heartwood contains higher concentrations of resin acids than the sapwood [15], and the bark contains a high concentration of resin acids [31]. Therefore, the resin acid concentration within wood not only depends on the tree species, but also depends on the amount of heartwood or bark the tree produces. The significance of these variations will become more apparent throughout this section.

The industry primarily responsible for discharging resin acids into aquatic environments is the pulp and paper industry. During the conversion of wood into pulp, resin acids are released with other wood extractives into the mill's process water. Some of this process water ultimately becomes pulp mill effluent after biological treatment. Release of resin acids occurs during the

Pimaranes



Abietanes

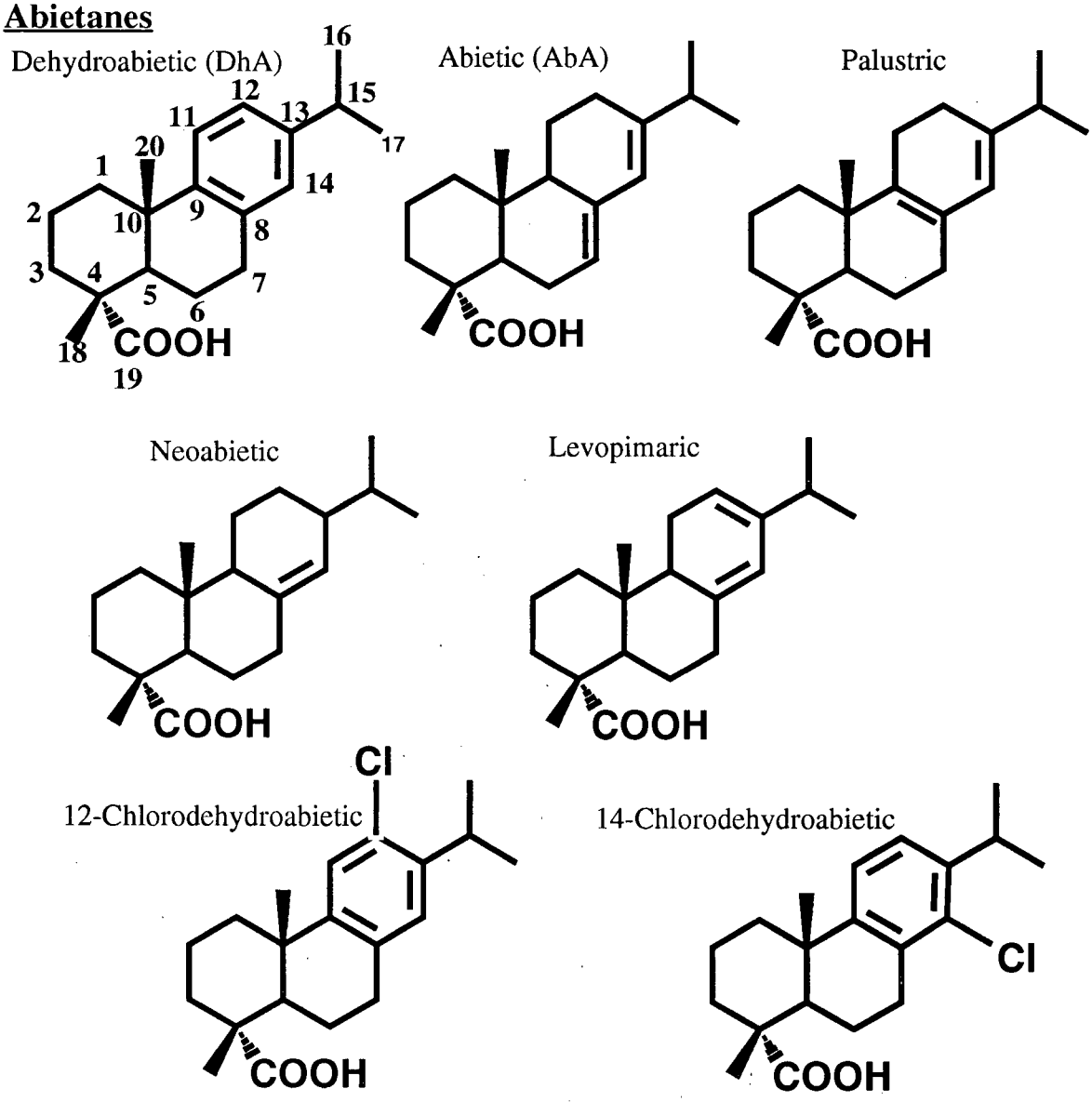


Figure 1: The structure of ten resin acids belonging to either the pimarane or the abietane family. The carbon skeleton numbering is presented on DhA.

debarking [31], pulping, and bleaching stages of the process. The structure of eight of the common resin acids found in pulp and paper mill effluents [49], plus two chlorinated derivatives of dehydroabietic acid (DhA) are shown in Figure 1. These chlorinated derivatives, such as 12-chlorodehydroabietic acid (12-Cl-DhA), 14-chlorodehydroabietic acid (14-Cl-DhA), and 12,14-dichlorodehydroabietic acid, which are most likely formed during the bleaching process, have also been found in pulp and paper mill effluents. The amount of resin acids released into the process water is dependent on several factors. The resin acid content in the starting wood, as previously stated, varies between tree species and among the same tree species and declines as the wood or woodchips are aged outside. The amount also depends on the method of pulping, such as the kraft process, sulphite process, chemi-thermomechanical pulping (CTMP), thermomechanical pulping (TMP) or mechanical pulping method. Additional mill parameters, including water use and hours of operation, also affect the concentrations of resin acids discharged. Therefore, the types of and quantities of resin acids released into the waste stream will vary. They will vary between pulp mills, but also within the same pulp mill during a 24-hr period.

Resin acids can cause problems to both the operation of pulp and paper mills and quality of the aquatic environment, once released as a component of the effluent. Resin acids in the process stream will become more problematic to the operation of pulp and paper mills, as the industry progresses toward a closed-mill system. In a closed-mill system, no liquid stream is released as effluent, so this type of mill system recirculates water. The excess whitewater created during the laying down and drying of the pulp mat is an economical choice for recirculation. The paper machine uses the second largest amount of water in pulp and paper mills, next only to the bleaching stage [37]. Few chemicals are added to the whitewater stream which would interfere with the paper process, or biological treatment if required. Although recirculation of whitewater reduces water consumption, accumulation of extractives, such as resin and fatty acids, to

concentrations in excess of 200 mg/l have been demonstrated at lower water consumption rates [16]. These high concentrations of extractives present a problem to the papermachines, which can tolerate resin and fatty acid concentrations of only 12 to 15 mg/l [17]. Resin acids are included within a broad group called "detrimental substances", which interfere with the papermaking process by affecting the activity of the cationic retention aides required for the formation of uniform paper [46]. These "detrimental substances" cause "pitch" deposits on the paper, which decreases the paper strength, brightness, aesthetics and papermachine runnability [53]. Because the resin and fatty acid levels exceed the tolerated levels for the papermachines, an internal treatment system for the whitewater will be required. Operation of an internal whitewater treatment system, will have the additional challenges of higher contaminant levels and a higher treatment temperature. These additional challenges are discussed in more detail below in this section.

Besides being problematic to the papermaking process, resin acids are a threat to the aquatic environment when released as a component of the pulp and paper mill effluent. Resin acids are highly toxic compounds and have a 96-hour LC_{50}, also termed acute toxicity, of 0.4 to 1.1 mg/l in juvenile rainbow trout [27]. These compounds are the primary source of toxicity in pulp mill effluents [25, 26, 27, 31, 39]. Isopimaric acid (IpA) has been shown to be the most toxic constituent of both mechanical and kraft mill effluents. Fish exposed to 0.8 mg/l and less than 2 mg/l of IpA had a median lethal survival time of 500 minutes for rainbow trout and coho salmon, respectively [25, 27]. Besides being highly toxic, resin acids persist in the natural waters. Several reasons explain the persistent nature of resin acids in the environment. Their hydrophobic nature makes them less soluble, and thus less readily available for biodegradation, than hydrophilic compounds in natural waters. In addition, the structural components of resin acids such as rings, double bonds, tertiary carbon atoms and an aromatic ring for DhA make them

more recalcitrant than non-saturated chained alkyl hydrocarbons. The persistence of resin acids in anaerobic marine and freshwater enrichment cultures over a six month period, provides evidence of the recalcitrance of these compounds under those conditions [33]. Also, IpA has been shown to be more recalcitrant than other resin acids in both treatment systems and natural waters [14]. Dellinger [12] summed up the environmental concern posed by resin acids by documenting untreated pulp mill effluents that exceeded the acute toxicity level by up to ten times. Because of the toxic and persistent nature of resin acids, mill process streams are treated before being discharged into the aquatic environment.

The pulp and paper industry typically uses one of two microbial methods, activated sludge or aeration lagoons, to eliminate the toxicity associated with resin acids found in pulp and paper mill effluents. These methods effectively eliminate resin acid toxicity from many different types of mill effluents [29, 41, 56]. Anaerobic biological treatment is another means of removing resin acids from the effluent, but this is sometimes inadequate [30]. As previously discussed, the pulp and paper industry is progressing towards internal treatment of process streams, such as the whitewater stream. Since treatment of this stream has the additional challenge of being at a higher temperature and having higher concentrations of contaminants, research into the operation of such treatment systems is required. Several studies, have investigated the operation of different, biological treatment systems designed to treat highly contaminated effluents at high temperatures [17, 18, 29, 48]. Lo *et al.* [29] showed continued removal of nearly 100% of high concentrations of resin and fatty acids as the temperature of the chemostat was increased from 20°C to 50°C in 10°C increments. Johnson and Hall [17, 18] investigated the effectiveness of high temperature treatment of an artificially created, high-strength, whitewater process stream using a laboratory scale sequencing batch reactor (SBR) which was operated at 5 temperatures. The SBR effectively removed 100% of the resin acids up to a temperature of 40°C. When the SBR

was operated at 45°C and 50°C, only partial removal of resin acids occurred. Since this SBR was the basis of this thesis study, the operation of the system is discussed below in Materials and Methods. Tardif and Hall [48] showed effective removal of resin and fatty acids from the same high-strength whitewater up to temperatures of 55°C when a combined ultrafiltation-SBR system was used.

Although biological treatment can reduce resin acid concentrations to sublethal levels, a study showed the effectiveness to be only 25-50% in some cases [49]. The potential exists therefore, for treated effluents to contain resin acids at concentrations that exceed the acute toxicity levels for fish. A general inadequacy of the biological systems to remove resin acids is one explanation for this release of excess resin acids into the environment. Failure of functioning biological systems, which effectively remove resin acids is another. System failures may occur for several reasons. For example, common abnormalities in the operational process may cause fluxes in the waste water components, that may result in toxicity shocks to the treatment system. Also, the general lack of knowledge of basic parameters for optimal resin acid degradation may limit optimum removal ability.

Erratic changes, termed spikes, in pH or resin acid concentration occur in treatment systems as a result of these operation abnormalities and process fluxes. Erratic changes in pH generally result from the lack of pH control, or when a spill of black liquor (alkali) or chlorinated filtrate (acid) occurs. Spikes in resin acid concentrations result from changes in the wood species used, the pulping method or the parameters of operation. Although these spikes are frequent occurrences in biological treatment systems, little is known about the resilience of the microbial community to these spikes. Lo *et al.* [29] showed that the removal of resin and fatty acids from a high-strength, recycled, CTMP wash effluent was not decreased significantly at pHs between 5 and 9. However, the pH was not rapidly changed in this study. George and Gaudy [13] showed

rapid pH changes of up to one unit can be tolerated without disrupting the efficiency of biological substrate removal of glucose in a laboratory scale continuous-flow treatment system.

Treatment systems also release excess resin acids because the original design did not plan for elimination of acute toxicity, mainly caused by resin and fatty acids. This changed when federal legislation was passed in the early 1970's requiring the elimination of this acute toxicity. Implementation of biological treatment systems has reduced this toxicity as discussed above. To further optimize resin acid removal, basic research on individual microbial isolates still needs to be done. Although basic research on the microbial biodegradation of resin acids started over 40 years ago, still little is really known. From 1956 to 1973, six bacterial isolates were reported to grow on the resin acids, abietic acid (AbA), DhA or their derivatives as a sole organic carbon source [5, 10, 11, 28, 40]. Delporte and Daste [11] first reported that an isolate from a pine forest floor, *Flavobacterium resinovorum,* was capable of growing on AbA as a sole carbon source. Raynaud and Daste [40] demonstrated the above isolate and a second bacterium, *Pseudomonas resinovorans,* were both capable of using artificial resins. Cross and Myers [10] followed by isolating a different soil bacterium, *Alcaligenes* sp., which used AbA. In the same year, Levinson and Carter [28] reported an *Arthrobacter* sp., isolated from lodgepole pine, which grew on methyl dehydroabietate. Biellmann et al. [5] isolated two additional bacterial strains, *Pseudomonas* sp. and *Alcaligenes eutrophus*, which grew on DhA, as a sole organic carbon source. Later in 1989, Cote [9] reported *Bacillus psychrophilus* transformed, but did not grow on DhA.

Some of these initial studies provided valuable information about possible resin acid degradation pathways. Cross and Myer [10] obtained three biotransformation products of AbA, by growing *Alcaligenes* sp. on succinate and then resuspending the cells in phosphate buffer and AbA. These products had either a hydroxyl group on the C-5, a hydroxyl group on the C-7 and a

hydrogen on the C-8, or the product was an epoxy-γ-lactone formed from an oxygen atom present as an epoxide ring between C-15 and C-2, or C-15 and C-6. Biellmann *et al.* [4 , 5, 6] studied the degradation pathway of DhA using two different bacteria. In the first study, *Flavobacterium resinovorum* [11], transformed DhA into a decarboxylated ketone [6]. A second study [4] provided more evidence for the degradation pathway using nutrient deficiency or inhibitors to cause different DhA intermediates to accumulate, including ring cleavage intermediates. Using these intermediates a hypothetical pathway was proposed (Fig. 2). In a third study, *Pseudomonas* sp. and *Alcaligenes eutrophus* [5] yielded additional intermediates of DhA. These intermediates confirmed the pathway proposed from the first study (Fig. 3), in which enzymatic hydroxylation of a ring carbon was followed by oxidation to a ketone. The only difference was the sole hydroxylation of C-7, instead of C-3. Levison [28] obtained different degradation intermediates from *Arthrobacter* sp. biodegradation of a methyl dehydroabietate. One of the metabolites was DhA. A second one, 3-oxodehydroabietic acid, could be an intermediate between the Biellmann pathway and the Brannon pathway discussed below.

Several studies have also investigated fungal biotransformation of resin acids. Brannon et al. [7] showed *Corticium sasakii* transformed different methyl dehydroabietanes by hydroxylating the C-3 to form an alcohol, and then hydroxylating a second carbon on a different ring to form a diol. If the C-7 contained a double bonded oxygen, then the second hydroxylation occurred at C-6. A series of resin acid transformation studies by Kutney et al. [19, 20, 21, 22, 23, 24] were done by growing *Mortierella isabellina* in dextrose yeast extract broth supplemented with a specific resin acid. *Mortierella isabellina* transformed resin acids by first hydroxylating the C-2 position, then hydroxylating either the C-15 or C-16 for abietanes such as DhA, AbA and 14-Cl-DhA. *Mortierella isabellina* similarly transformed 12-Cl-DhA and 12,14-dichlorodehydroabietic acid except the C-16 was not hydroxylated. *Mortierella isabellina* transformed IpA by

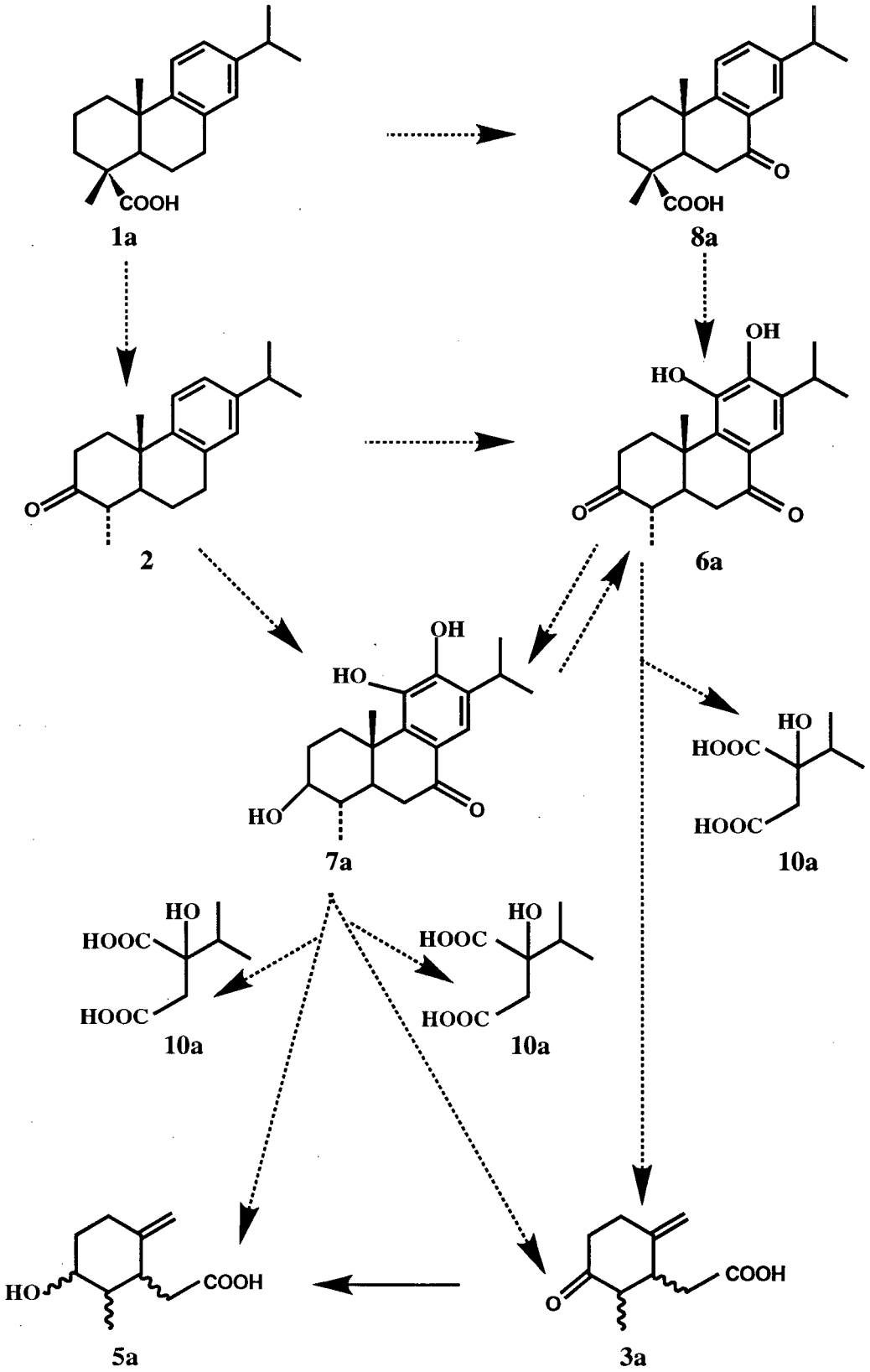


Figure 2: The proposed degradation pathway for DhA based on *Flavobacterium resinovorum;* numbering on metabolites taken directly from article [4].

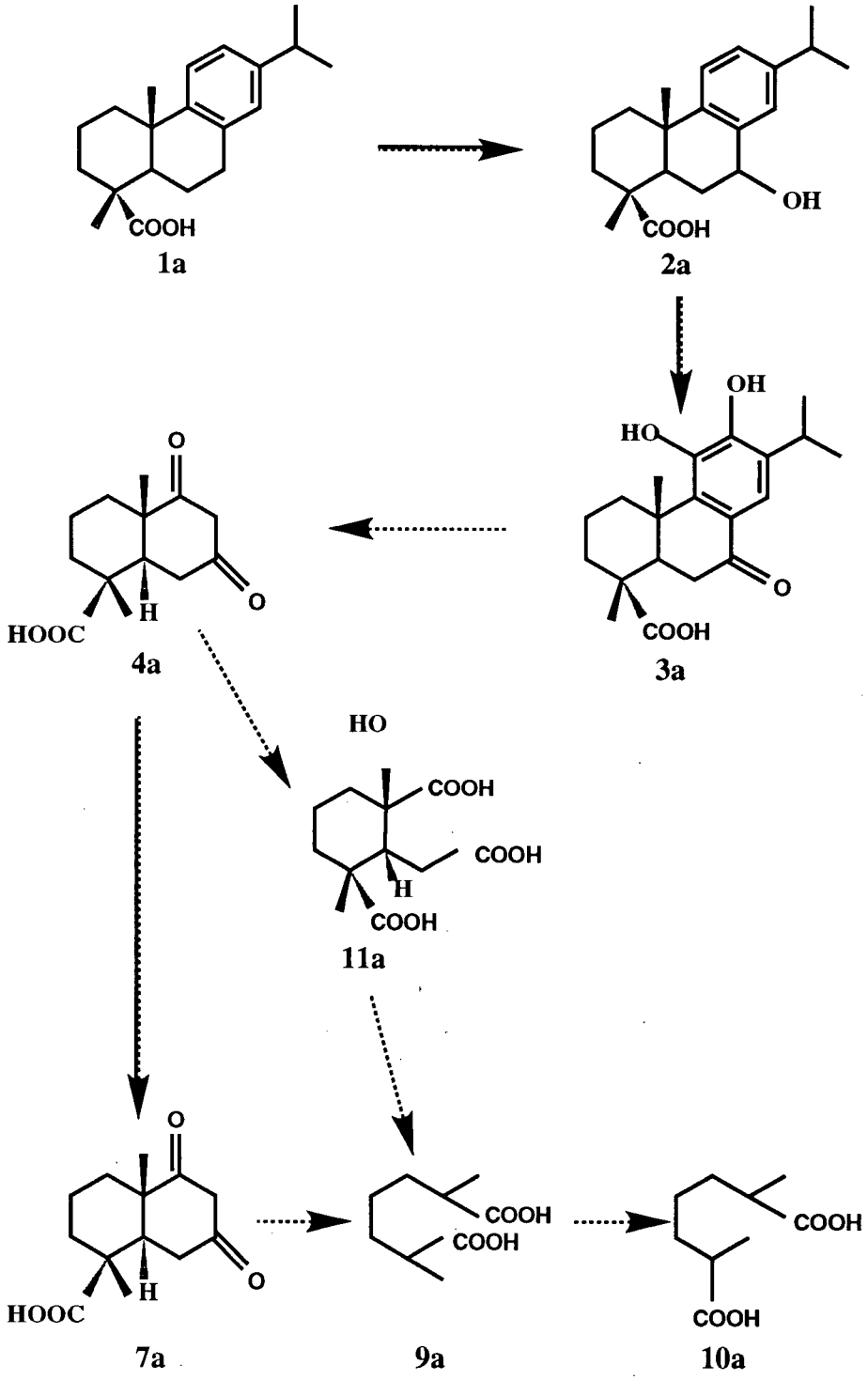


Figure 3: The revised degradation pathway for DhA based on *Pseudomonas* sp. and *Alcaligenes eutrophus;* dashed lines hypothetical pathway; solid lines, proved pathway; numbering on metabolites taken directly from article [5].

hydroxylating the C-2, both the C-15 and the C-16 or all three carbons. After the initial hydroxylations, no further transformation occurred. Most of these hydroxylated metabolites had a much lower acute toxicity to sockeye salmon fry or *Daphnia pulex,* than the original parent resin acid [42]. Interestingly, the IpA study showed that, of five fungi and six bacteria tested, only *Mortierella isabellina* transformed IpA, while none were able to grow or degraded IpA.

Fungal studies have also investigated the removal of resin acids from effluents and from wood before conversion to pulp. Spencer *et al.* [45] showed four fungal strains partially removed a resin acid mixture from a kraft mill effluent. Brush *et al.* [8] reported that wood chips treated with a commercial product Cartapip™, a non-staining *Ophiostoma piliferum* strain, reduced the total and specific resin acid content in the wood by 30 to 45%. However, the nonsterile control also reduced the total resin acid content by 45%. Wang et al. [52] demonstrated three *Ophiostoma* species, *O. ainoae*, *O. piceae* and *O. piliferum* (Cartapip™), and a *Lecythospora* sp. reduced resin acids in lodgepole pine blocks to varying extents depending both on fungal species and specific resin acid. These studies did not demonstrate the fate of these resin acids. The above research has provided valuable insight into resin acid degradation pathways and resin acid biotransformation, yet still little is known about resin acid specificity, resin acid metabolism and induction of resin acid degradation

Recently, three studies using resin acid-degrading organisms have begun to investigate resin acid specificity, resin acid metabolism and the induction of resin acid degradation. Mohn [32] isolated two bacterial strains, which grew on DhA as a sole organic carbon source, from the same laboratory scale SBR [17, 18] used for this thesis study. The strains grew on the individual abietanes: DhA, AbA and palustric acid, as sole organic substrates, but not on the pimaranes: IpA or pimaric acid (PiA). Growth on DhA by these strains correlated with DhA degradation and the conversion of DhA to protein was 0.25 to 0.30 g of protein per g of DhA. In cell suspensions,

DhA induced both DhA and AbA removal activity and this activity was heat-labile. Another study by Bicho *et al.* [3] described five bacterial strains, isolated from a bleach kraft mill effluent enrichment, which grew on DhA as a sole organic carbon source. Growth on DhA by three of the five strains correlated to almost complete DhA disappearance. In the presence of DhA, all five strains removed the abietanes: DhA, AbA, and 7-oxo-DhA, but did not remove the pimaranes: IpA, PiA and sandaracopimaric acid. All the strains removed 12-Cl-DhA, but not 14-Cl-DhA or 12, 14-dichlorodehydroabietic acid. Most recently, Morgan and Wyndham [35] also isolated thirteen AbA or DhA-degrading bacterial strains from a bleach kraft mill effluent. All thirteen isolates were shown to remove six resin acids, including IpA, DhA, AbA, PiA, to varying extents. The fate of these resin acids was not demonstrated, except for one instance. One strain was shown to grow and remove AbA as a sole organic carbon substrate.

Very few studies have investigated the substrate range of resin acid-degrading organisms. *Flavobacterium resinovorum* [11] used the wood sugars: arabinose, xylose, glucose, galactose and maltose. The two DhA-degrading isolates [32] also oxidized several wood sugars: arabinose, cellobiose, galactose and grew on xylose, acetate, benzoate, and pyruvate. The above studies have begun to provide valuable knowledge about microbial specificity for resin acids, metabolism of abietane-degrading organisms and specificity for other substrates. At the same time, no organism capable of growing on a pimarane as a sole organic substrate has been reported.

Despite the lack of evidence for pure cultures transforming pimaranes, aerobic biological treatment systems can effectively remove resin acids, including pimaranes. One example was the SBR previously discussed, which efficiently removed several resin and fatty acids, including pimaranes, from a high-strength, paper mill process stream [17, 18]. Because the SBR was operated at 5 different temperatures, it provided a unique opportunity to investigate resin acid-degrading organisms, but also to investigate a dynamic resin acid-degrading community.

Therefore this SBR became an integral part of this thesis research and the operational parameters of this system are described in the Materials and Methods.

The focus of this thesis was to study the resin acid-degrading community of the SBR. Four main questions were to be investigated. 1) Since no IpA-degrading organisms have been isolated, is IpA fortuitously degraded by abeitane degraders, or do IpA degraders exist? 2) Did increasing the operational temperature of the SBR change the ecology of the resin acid-degrading community in terms of the species present, their resin acid specificities, their temperature ranges and their substrate ranges? 3) Was induction by one or more resin acid in the SBR required to obtain biodegradation of all resin acids? 4) Was the resin acid-degrading community of the SBR resilient to naturally occurring spikes in pH and resin acid concentration?

MATERIALS AND METHODS

1. Culture Media and Stock Cultures

Two different media, enrichment medium and culture medium, were used in this study. The enrichment medium was used for incubation of SBR biomass and for the isolation of strains from the SBR. The culture medium was used for all subsequent experiments, except where noted. The enrichment medium [32], a basal mineral medium, contained the following final concentrations (per liter): 0.33 g of K_2PO_4, 0.16 g of KH_2PO_4, 0.53 g of NH_4Cl, 0.10 g of $NaSO_4$, 0.18 g of $MgCl_2$, and 0.022 g of $CaCl_2 \cdot H_2O$. To make the medium, the first four compounds, the major salts, were dissolved in 90% of the final volume, and the pH was adjusted to 7.0 with HCl or NaOH. The last two salts were dissolved in 10% of the final volume. The two solutions were autoclaved separately and mixed after cooling. Vitamins, trace elements [43] and 10 mg of $FeSO_4 \cdot H_2O$ per liter were added from filter-sterilized stock solutions. The vitamin stock solution was made as previously described [32]. The culture medium [32] consisted of the above mineral medium, except the phosphate buffer was increased from 3 mM to 10 mM and the minor salts were decreased to 0.030 g of $MgCl_2$, and 0.003 g of $CaCl_2 \cdot H_2O$ per liter. Solid media contained 1.5% Select Agar (BBL, Cockeysville, Md.). Stock cultures were maintained on tryptic soy broth (Difco, Detroit, Mich.) with 1.0 g of pyruvate per liter added.

Resin acids were purchased from Helix Biotechnologies, Richmond, BC, Canada. The manufacturer-reported purities of analytical-grade resin acids were 99% for IpA and DhA, 96% for AbA, 92% for the mixture of 12,14-chlorodehydroabietic acid (Cl-DhA), and 88% for PiA (with the remaining 12% being sandaracopimaric acid). The purities of technical-grade resin acids were 90% for IpA, 50% for DhA (ICN Biochemical, Cleveland, Oh.), and 70% for AbA. The resin acids were dissolved in a NaOH solution of twice the molar concentration of the resin acid,

and then filter-sterilized before addition to autoclaved media. Insoluble suspensions of the resin acids were present in the cultures.

Most other organic substrates were added to autoclaved culture media from filter-sterilized stock solutions. Stock solutions of benzoate (99%, Aldrich, Milwaukee, Wis.), 3-chlorobenzoate (3-CB) (99%, Aldrich), phenol (99%, BRL, UBC, Vancouver, Canada) or pentachlorophenol (PCP) (99%, Helix Biotechnologies) were made in NaOH as described above. Stock solutions of betulin (97%, Aldrich), biphenyl (99%, Aldrich), camphor (96%, Aldrich), β-citronellol (95%, Aldrich), hexadecane (99%, Fisher, Edmonton, AB, Canada) napthalene (99%, Aldrich), or β-sitosterol (99%, Sigma, St. Louis, Mo.) were made by dissolving the substrate in acetone. Those substrates, which were dissolved in acetone (which was not used as a growth substrate), except for camphor, citronellol and hexadecane formed an insoluble suspension in the media. Stock solutions of all other substrates were made by dissolving the substrate in distilled water. The wood sugars were added to autoclaved culture media from autoclaved stock solutions. Linoleic acid (95%, Sigma), a liquid, was filter-sterilized and added directly. Palmitic acid crystals (J.T.Baker, Phillipsburg, NJ) were added directly without sterilization (growth never occurred in uninoculated control cultures).

Glass tubes, used in resin acid experiments, were cleaned by soaking overnight in a 5% Contrad 70 (Baxter, Toronto, ON, Canada) solution, rinsing with distilled water, and finally rinsing with ethyl acetate.

2. Operation and Sampling of the Sequencing Batch Reactor (SBR)

The treatment system studied as a part of this research was a laboratory scale (10-liter) SBR used to treat a high-strength, process stream from a paper mill [17, 18]. The SBR was operated by Rhiannon Johnson for her Masters thesis project in the Department of Civil

Engineering, University of British Columbia. The make-up of the influent and operational conditions relevant to this study are included below. The influent for this treatment system contained both plug screw pressate (containing resin and fatty acids) from a thermomechanical pulp mill (Howe Sound, B.C.) and evaporator bottoms (containing high levels of organic and inorganic matter) from a closed-cycle chemi-thermomechanical pulp mill (Meadow Lake, Sask.). The total resin acid concentration in the influent was typically ranged from 13 to 30 mg per liter, including 5 to 10 mg of DhA, 2 to 6 mg of AbA, and 3 to 8 mg of IpA per liter of influent. The influent also contained fatty acids with the two most abundant fatty acids being linoleic acid and palmitic acid at concentrations ranging from 4 to 8 mg and 3 to 13 mg per liter, respectively. The SBR was set at five different operational temperatures, 20°, 30°, 40°, 45° and 50°C. The SBR was on a 24-h cycle with a hydraulic retention time of 48 h and a solids retention time of approximately 20 days. Samples from the SBR were taken just before the end of the aeration period of a 24-h cycle, 21.5-22.5 hours post-feeding. The samples were immediately used for tests.

3. Biomass Resting Cell Assays

To determine if high concentrations of resin acids or fluxes in pH affected the ability of the biomass to remove resin acids from the SBR, resting cell assays were set up. Erylenemyer flasks (125 ml) contained 12.5 ml of enrichment medium to which 12.5 ml of concentrated SBR biomass was added. This concentrated biomass was obtained by taking a 600 ml sample of the SBR, settling the biomass, and decanting the supernatant. This left about one-third of the initial volume and constituted the concentrated biomass used in these experiments. For the pH experiments, the assays were set up slightly differently to obtain reproducible pHs. To each of five flasks containing 37.5 ml of enrichment medium, 37.5 ml of concentrated biomass was added. The

enrichment medium was supplemented with Tris and MES to final concentrations of 10 μM for buffering capacity. The pH of the medium was adjusted to 5.0, 6.0, 7.0, 8.0, or 9.0 using filter sterilized 4 N HCl or 4 N NaOH, then the volume was split into three 25 ml aliquots, and readjusted to the final desired pH just before the test started. The control flasks contained 25 ml of enrichment medium, but no biomass. Each experiment was started, by the addition of a mixture of three resin acids to the flasks, which were then incubated at 30°C on a shaker (280 rpm). The mixture contained technical-grade resin acids in proportions equal to those in the SBR, 25%:50%:25% for IpA-DhA-AbA.

4. Most Probable Number (MPN) Method

Enumeration of the resin acid-degrading population was performed by the most probable number method [2]. Prior to each dilution and inoculation, samples were vigorously shaken 20 times and vortexed for 30 seconds to disperse cell aggregates. Serial dilutions of the SBR sample (Methods and Materials, section 2) were made in 4.5-ml dilution blanks containing only the major salts of the enrichment medium. An inoculum of 0.3 ml of the specified serial dilution was added to each of five replicate tubes which contained 2.7 ml of enrichment medium supplemented with three, technical-grade, resin acids at the same concentrations as in the SBR. The final concentration of each tube was 25 μM of IpA, 50μM of DhA and 25 μM of AbA, except for one test that contained only 100 μM of IpA. The tubes were incubated stationary at the operational temperatures of the SBR, and weekly monitored for growth and resin acid disappearance until both ceased. Growth was evaluated by microscopy and resin acids were quantified by gas chromatography analysis (GC). For each resin acid, the tubes were scored positive when complete disappearance of that resin acid occurred. The most probable number determination was then calculated as previously described [2].

5. Enrichment and Isolation Techniques

The endpoint dilution method and the selective enrichment method were used to isolate resin acid-degrading organisms from the SBR. The organisms were isolated on their ability to grow on one of the three primary SBR resin acids, IpA, DhA or AbA, as a sole organic substrate. For the endpoint dilution method, the greatest dilution of MPN tube (Results, section 1.C) exhibiting both growth and disappearance of a specific resin acid was used to isolate either AbA-degrading, IpA-degrading or DhA-degrading organisms. This culture was streaked directly on solid medium containing 100 μM of that specific, analytical-grade, resin acid (IpA, DhA or AbA) to obtain isolated colonies. A second way colonies were obtained using the endpoint dilution method was by the addition of a 0.1% inoculum from the same MPN tube to 3.0 ml of enrichment medium containing 100 μM of that resin acid. The tube was then incubated stationary at the temperature of the SBR. After growth and depletion of the specific resin acid, the enrichment culture was consecutively transferred twice to fresh enrichment medium which contained the same initial resin acid concentration. The culture was then streaked on solid medium containing 100 μM of the specific, analytical-grade resin acid to obtain isolated colonies.

For the selective enrichment method, a 0.1% inoculum from the SBR (Materials and Methods, section 2) was added to three tubes each containing 3.0 ml of enrichment medium. The enrichment medium contained 100 μM of one of the three, technical-grade resin acids (IpA, DhA or AbA). The cultures were incubated stationary at the operational temperature of the SBR (20°, 30°, 40°C, 45°, or 50°C). After growth and disappearance of the specific resin acid had occurred, the enrichment culture was consecutively transferred twice to fresh enrichment medium which contained the same initial resin acid concentration. Then, the culture was streaked on solid medium containing 100 μM of the specific analytical-grade resin acid to obtain isolated colonies.

6. Physiology and Characterization of Substrate Use by Isolates.

To determine the substrate range of the isolates, 16 x 100 mm glass tubes or glass tubes with Teflon-lined screw-caps were used. Each tube contained 2.5 ml of culture medium supplemented with the analytical-grade substrate to be tested. A tube with a Teflon-lined screw-cap was used if the culture was to be extracted to determine substrate disappearance by GC analysis. Each tube was inoculated with a late-log phase culture of the desired resin acid-degrading strain, then incubated stationary (except as noted) for 14 days at the isolation temperature of the specific organism (20°, 30°, or 40°C). Growth on sugars, acetate, ethanol, methanol and pyruvate was quantified by optical density (OD_{610}) in a Cole-Parmer colorimeter (Cole-Parmer, Niles, Ill.). For all other substrates, including resin and fatty acids, growth was evaluated by microscopy, and substrate disappearance was quantified by the specified GC analysis method. Microscopy was used because the insoluble substrates interfered with measurement of optical density. The maximum growth temperature of a strain was determined in culture medium supplemented with the resin acid used for that strain's isolation. Cultures were incubated shaking, rolling or stationary at the following temperatures; 20°, 25°, 30°, 37°, 40°, 45°, or 50°C. The strains were tested for anaerobic use of glucose under fermentative conditions or with 13 mM nitrate using the method of Mohn [32].

Oxidation of a battery of substrates was tested using GN Microplates (Biolog, Hayward, Calif.). Catalase, oxidase, and Gram reactions were tested on colonies grown 24 to 48 h on tryptic soy broth (Difco, Detriot, Mich.) supplemented with 1.5% Select Agar (BBL, Cockesyville, Md.) and 1.0 g of pyruvate per liter. Catalase tests were performed on a glass slide with a drop of 3% H_2O_2. Oxidase tests were performed by directly dropping oxidase reagent, 1% N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride solution, (Sigma, St. Louis, Mo.),

on the colony. Gram stains were performed using the reagents and method provided with the BBL Gram Stain Reagents and Kit (Becton Dickenson and Co., Cockesyville, Md.).

7. Growth of IpA-Degrading Strains

For growth curve experiments, IpA-degrading strains were grown in individual glass tubes with Teflon-lined screw-caps. Individual cultures were required at each sampling time point because the entire culture was used to quantify either IpA or protein. The tubes contained 1.0 or 5.0 ml of culture media supplemented with analytical-grade IpA to a final concentration of 100 μM, unless other wise stated. For all growth experiments, analytical-grade resin acids were used. For growth yield experiments, the strains were grown in 16 x 150 mm glass tubes containing 12 ml of culture medium and various amounts of IpA, 0, 0.73, 1.46, and 2.19 mg (0, 200, 400, and 600 μM). For carbon mass balance determinations, the strains were grown in 26-ml butyl rubber stoppered glass tubes containing 10 ml of culture medium supplemented with IpA to a final concentration of 200 μM. For cell suspension experiments, IpA-degrading strains were grown in 250 ml Erlenmeyer flasks containing 40 ml of culture medium supplemented with either 400 μM of IpA or 0.3 g of glucose per liter. To grow the cultures, a 1% inoculum of the desired IpA-degrading strain as described below, was added to media and then incubated on a tube roller (32 rpm) for the culture tubes or on a shaker (180 rpm) for the culture flasks. The cultures were incubated at 30°C for strains IpA-1 and IpA-2 and 40°C for IpA-13, unless otherwise stated.

The following protocol was adopted to insure all cultures were inoculated with a late-log phase cultures of IpA-1 and IpA-2, and a late stationary-phase culture of IpA-13. For IpA-1 and IpA-2, a preinoculum culture was made by inoculating a colony of the desired strain into 3.0 ml of culture medium containing 200 μM IpA. Next, 1% of this late-log phase preinoculum culture was

transferred to fresh culture medium containing the same concentration of IpA. This was then used as inoculum when it reached late-log phase based on predetermined growth curves. For IpA-13 the inoculum was prepared by adding 30 µl of the frozen stock culture into 3.0 ml of culture medium containing a final concentration of 200 µM IpA. When the culture reached late stationary phase, as indicated by a cocci-rod morphology, the inoculum was used.

8. Photomicrograph Preparation

At each time point, 900 µl of IpA-13, which was grown in culture medium supplemented with 200 µM IpA, was transferred to an microcentrifuge tube to which 100 µl of 33% formaldehyde was added as a preservative. The cells were concentrated by pelleting (20 min at 16,0000 x g in an Eppendorf benchtop microcentrifuge) and then suspended in 20 to 100 µl of culture medium. A drop of the concentrated culture was immobilized on a glass microscope slide previously coated with 1% Select Agar (BBL, Cockeysville, Md.), and a cover slip was placed over top. The photomicrographs were taken with a MC 80 microscope camera mounted on an Axiolab Zeiss microscope (Carl Zeiss, Oberkocen, Germany) using Kodak technical panfilm (ester-AH base, TP 135-36, ASA 25).

9. Cell Suspension Assays

Cell suspensions were prepared based on the method of Mohn [32]. Cultures were grown to late-log phase as previously described (Section 7), then placed on ice. Cells were harvested by centrifuging at 23,500 x g (4 - 8°C, 20 min), then washed in an equal volume of cold culture medium which did not contain trace elements, vitamins or $FeSO_4$. The cells were centrifuged a second time and finally suspended in a lesser volume of the same cold medium. In a 7°C coldroom, 500 µl of concentrated cells were added to individual, sterile, glass tubes with Teflon-

lined screw-caps, followed by the addition of 1 µl of a 50 mg/ml chloramphenicol solution where indicated. Lastly, 500 µl of a 400 µM solution of the desired analytical-grade resin acid was added (final resin acid concentration, 200 µM). Killed cell suspensions were prepared by boiling the cells for 10 min prior to the addition of chloramphenicol or resin acids. Cell suspensions had a final OD_{610} of 0.37 to 0.77 (using a Varian-Cary 1E UV-Visible Spectrophotometer, Mountainview, Calif.), which corresponds to 82-480 µg protein per ml of culture. The individual tubes were incubated on a tube roller (32 rpm) at 30°C, and samples were frozen at each time point. Samples were then quantified for resin acids using GC analysis.

10. Gas Chromatographic Analysis of Resin Acids, Fatty Acids, Biphenyl, Napthalene, Betulin, β-sitosterol, Camphor, β-citronellol, and Hexadecane

Resin acids, fatty acids, biphenyl, napthalene, betulin, camphor, β-citronellol, hexadecane and β-sitosterol were quantified by gas chromatography. Resin and fatty acids were extracted and quantified as previously described [32]. Samples to be extracted were either taken from vortexed cultures (500 µl) or were whole cultures in tubes with Teflon-lined screw-caps. The Teflon-lined screw caps allowed the samples to be extracted in the original sample tube. An internal standard of 50 µM 12, 14-dichlorodehydroabietic acid was added to each sample, which was then extracted twice with equal volumes of ethyl acetate. Extracts of each sample were pooled, dried over anhydrous $NaSO_4$ and then transferred to auto-sampler vials. The resin or fatty acids were derivatized by bubbling CH_2N_2 into auto-sampler vials containing the sample. CH_2N_2 was produced by passing nitrogen through a special apparatus to which 0.5 g diazald (Sigma), 2 ml methanol, 4 ml ether was added. To initiate the reaction, which produced the CH_2N_2, 0.5 ml of 11 N KOH was added.

For analysis of biphenyl and napthalene, an internal standard of 50 ppm 3-chlorobiphenyl was added to each 2.5-ml sample in a tube with a Teflon-lined screw-cap. The sample was extracted twice with 3 ml of hexane. Extracts of each sample were then pooled.

For analysis of betulin and β-sitosterol, 1.5 ml of the following solvents were added to the samples: hexane was added to β-sitosterol samples while acetone-hexane (1:2) was added to betulin samples. In addition to the solvent, 2.5 ml of 0.1 M K_2CO_3 and 200 µl of acetic anhydride was added to each of the 2.5-ml samples. These mixtures were shaken and vented immediately. This process was repeated until acetylation went to completion, as evident by the slowing of bubble production. The process allowed simultaneous extraction and acetylation of betulin or β-sitosterol. The solvent phases were removed and the aqueous phases were extracted a second time with hexane for β-sitosterol samples and acetone-hexane (1:2) for betulin samples. Extracts of each sample were pooled, then combined with 3.0 ml of 0.05 M K_2CO_3 to remove any residual acetic anhydride from the solvent phase.

For analysis of camphor, citronellol and hexadecane, the entire 2.5-ml culture was extracted twice with 1.5 ml of hexane. Extracts of each sample were pooled, then combined with 3.0 ml of 0.05 M K_2CO_3 to remove any residual acetic anhydride from the solvent phase.

The above extracts were analyzed by injecting 2.0 µl on a Hewlett-Packard 5890 Series II gas chromatograph equipped with an autoinjector, a Hewlett-Packard, HP-5 column (25 m by 0.32 with a 0.17-µm solid phase), and a flame ionization detector (FID). The injector and detector were set at 260 and 300°C, respectively. The carrier was H_2 at a constant flow rate of 2.4 ml/min. The temperature programs were as follows: Resin and fatty acids: 2 min at 70°C, 30°C/min to 195°C, 0.6°C/min to 200°C, 20°C/min to 280°C and 2 min at 280°C. Biphenyl and

naphthalene: 2 min at 80°C, 7.0°C/min to 178°C, 30°C/min to 280°C and 2 min at 280°C. Betulin, hexadecane and β-sitosterol: 2 min at 80°C, 30°C/min to 310°C, and 5 min at 310°C. Camphor: 2 min at 80°C, then 4.0°C/min to 98°C. Citronellol: 2 min at 80°C, 15°C/min to 125°C, 30°C/min to 310°C, and 3 min at 310°C. For resin acids, the extraction efficiencies were 90 to 104% based on at least triplicate determinations. The GC detected resin acids down to 2 μM. The standard curve for resin acids was linear between 5 μM and 200 μM.

11. Benzoate, 3-Chlorobenzoate (3-CB), Phenol and Pentachlorophenol (PCP) Analysis

Benzoate, 3-CB, phenol and PCP were quantified by high performance liquid chromatography (HPLC). For phenol and PCP, 0.5 ml of either sample was combined with 0.5 ml of methanol then filtered through a 0.45 μm filter prior to injection. Benzoate or 3-CB samples were prefiltered through a 0.45 μm filter before injection. Each sample (10 μl) was analyzed using a Hewlett-Packard 1054 HPLC equipped with an autoinjector, a reverse phase ODS Hypersil column (150 mm by 4 mm with a particle size of 5 μm), and a UV variable-wavelength detector. The column temperature was set at 30°C, and the flow rate was 1 ml/min. The elution buffer for benzoate and 3-CB was 0.5% phosphoric acid-methanol (45:55). The same elution buffer was used for phenol and PCP, except the ratios were 70:30 and 20:80, respectively. The detector was set at a wavelength of 230 nm for benzoate and 3-CB, 271 nm for phenol, and 304 nm for PCP.

12. Protein Assay

For the protein assays, cells were collected either by centrifugation for strains IpA-1 and IpA-2, or by filtration for IpA-13, because IpA-13 did not pellet well. For IpA-1 and IpA-2, individual protein samples were transferred to microcentrifuge tubes, pelleted (20 min at 16,0000

x g in an Eppendorf benchtop microcentrifuge), washed in the original volume of sterile saline, then pelleted once more as described above.

For IpA-13, individual cell samples from growth curves were collected on 150-mm diameter Cellulose Plus filters (MSI, Micron Separations Inc., Westboro, Mass.) by vacuum filtration using a Biorad 96-Well Dot Blot apparatus (Biorad, Mississauga, ON, Canada). The filters were air dried and individual filter dots, which contained one sample, were punched out and transferred to microcentrifuge tubes. The larger-volume IpA-13 samples, such as growth yield or carbon mass balance samples, were filtered onto individual 25-mm diameter cellulose acetate filters (0.45 μm, Sartorius, Gottingen, FRG) using a Millipore 25-mm diameter vacuum-filter apparatus (Millipore, Bedford, Mass.). The filters were then placed in glass tubes with Teflon-lined screw caps for cell lysis. Prior to sample collection, all filters were soaked in distilled H_2O and placed on the filter apparatus. Filtered samples were rinsed twice with equal volumes sterile saline to remove any extraneous matter.

For IpA-1 and IpA-2, cells obtained from growth curves were lysed by suspending the pellets in 500 μl of 1% SDS at room temperature. Cells of IpA-1 and IpA-2 obtained from growth yield, carbon mass balance determinations, and cell suspension experiments, were lysed by incubating at 95°C in 1 M NaOH (50-100μl) for 10 min. The samples in NaOH were then diluted ten-fold with sterile distilled water. IpA-13 cells were lysed by adding 200 μl of 0.5 M NaOH to the microcentrifuge tubes containing the hole-punched filters or by adding 2.0 ml to the glass tubes containing the 25 mm filters. The tubes were vortexed until the cells visibly detached from the filters. The tubes were then incubated for 10 min at 95°C. After incubation, the volume in the microcentrifuge tubes containing lysed IpA-13 cells was brought up to 1 ml with sterile distilled water.

Protein was quantified using the micro bicinchoninic acid method [38], or the bicinchoninic acid method (BCA) [44]. The micro bicinchoninic acid method and reagents (micro-BCA) were used for all growth curve samples. To the 500 µl IpA-1 and IpA-2 samples were added 500 µl of the micro-BCA reagent, which was made by combining MA:MB:$CuSO_4$ (4%) in the following ratio (50:48:2). For IpA-13, 1 ml of a slightly modified micro-BCA reagent (50:49.2:0.8) was added to 1.0 ml of sample. The reagent was modified because precipitation occurred when the normal ratio was used. The assays were incubated for 1 h at 60°C. The bicinchoninic acid method was used for all other protein assays of the three strains. In an ice bucket, the reagent was made by combining 50 parts bicinchoninic acid solution (Sigma) to 1 part $CuSO_4$ (4%), then 950 µl of the reagent was added to 50 µl of various dilutions of the sample. The assays were incubated for 30 min at between 37° and 45°C. The reactions were stopped by placing the tubes on ice. For both assays the reactions were read at wavelength of OD_{562} using a Varian-Cary 1E UV-Visible Spectrophotometer (Varian-Cary, Mountainview, Calif.). To calculate µg/ml protein, bovine serum albumin (Sigma) was used to run a standard curve. The standard curve for protein was linear between 0 µg/ml and 32 µg/ml for the micro-BCA assay and between 0 µg/ml and 20 µg/ml for the BCA assay.

13. CO_2 Assay

CO_2 assays were performed on cultures grown in 26-ml stoppered glass tubes, containing 10 ml of culture. Before assaying for CO_2, culture tubes were injected with 2 drops of a 4 N HCl, which resulted in a pH drop to 2.0 and the conversion of all the inorganic carbon to be released as CO_2. The culture tubes were equilibrated to room temperature prior to sampling. CO_2 was quantified by gas chromatography by directly injecting 100 µl of headspace sample on a Shimadzu GC-8A gas chromatograph equipped with a Haysep DB packed column (3.05 m by 3.2 mm) and

a thermal conductivity detector. For the CO_2 assay, the injector and detector were set at 140°C, the column was set at 100°C, and the carrier gas was He at 30 ml/min. To calculate CO_2, a standard curve was run, using standards made in N_2. Since the standard curve was linear, a one point calibration was used to calculate CO_2 values.

14. Dissolved Organic Carbon (DOC) Assay

Samples were filtered or centrifuged to remove suspended solids, then acidified to pH 2.0 using 4 M HCl and finally stored at 4°C for no more than 7 days. The acidification step converted all inorganic carbon to CO_2, making the total organic carbon values measured equivalent to the actual dissolved organic carbon in the liquid. The treated samples were assayed for total organic carbon according to a standardized method using a Shimadzu TOC-500 automatic carbon analyzer [1]. DOC supernatants were divided into high and low molecular-weight fractions, above and below 10 kD, using a Centricon-10 (Amicon, Beverly, Mass.) filter centrifuged for 1 h at 5,000 x g. These fractions were then assayed again for DOC as described previously.

15. Carbon Mass Balance Calculations

To obtained the biomass carbon value, grams of protein was converted to grams of dry cell assuming cells are 55% protein. The cell mass was finally converted to grams of carbon, assuming the cells are 60% carbon. The CO_2 value was calculated in atmospheres, converted to moles, then converted to grams of carbon. DOC was converted from ppm to grams, based on the known sample volume. IpA was converted from μM to grams, based on the known sample volume, then converted to grams of carbon based on the fact that IpA is 79.4% carbon. Values for net percent of initial carbon as biomass, CO_2, DOC and IpA were determined as values for

cultures grown on IpA less the corresponding values for inoculated control cultures having no IpA as substrates.

16. Biosurfactant Assay

To test for the presence of biosurfactant production, surfactant activity in terms of dynamic surface tension was measured by the maximum bubble pressure method [36]. The dynamic surface tension of a time course sample was immediately measured using an apparatus designed, operated and previously described by Alan Werker [55]. To measure the dynamic surface tension, sample tubes were immersed up to the tube's liquid volume in a 30°C water bath (400 ml), which was jacketed by a thermostated bath. Using the above apparatus, the maximum bubble pressure method was performed by introducing bubbles at a constant rate into the sample through a micropipette (~0.04 mm capillary diameter) at a depth of 1.27 cm. The depth of the micropipette was determined using a needle gauge micrometer with a measurement accuracy of 0.025 mm. The bubbles were formed at a constant pressure supplied by an air charged reservoir which was pressurized by displacement with deionized water from a constant head reservoir. Over a 10 to 20 min period, pressure-time values for all the bubble formation events were logged at 200 Hz directly onto a computer (12 bit A/D) through a calibrated differential pressure transducer which produced a 1 volt/cm H_2O signal. Once the raw data were reviewed, the representative data were converted to pressure plots. From these data plots, two types of measurements were chosen as indicators of dynamic surface tension. The first measurement was dynamic surface tension, σ_{20}, at a bubble age of 20 seconds. This was derived from a series of

back calculations based on the Laplace equation:

$$P_{max} = P - P_o = \frac{4\sigma\tau}{D} + \rho g h$$

The pressure in the bubble, P, required to produce an assumed spherical bubble is a maximum (P_{max}) when the emerging bubble becomes a hemisphere with the capillary's radius (D/2): where P_o is the atmospheric pressure, ρ is the liquid density, g the acceleration due to gravity which is constant and σ is the dynamic surface tension dependent on the bubble formation time, τ. The second measurement was t_x, in seconds, and describes the moment the surface tension initially changed.

RESULTS

1. The resin acid-degrading community of the SBR.

The first research objective was to examine how the resin acid-degrading microbial community of the SBR responded to rapid changes in either pH or resin acid concentration, and to increased operational temperatures. Two parameters were used to measure the response by the SBR community. Resin acid removal ability was used to measure the SBR community's response to rapid changes in resin acid concentration or pH, while population changes in the resin acid-degrading community was used to assess the effect of increased operational temperatures of the SBR. In order to assess population changes in the SBR community, the number of and the specificity of organisms growing on resin acids was measured.

1.A Effect of high resin acid concentrations on the biomass of the SBR.

To determine whether high concentrations of resin acids would have an inhibitory effect on the ability of the biomass to remove resin acids, biomass resting cell assays were set up with various concentrations (100, 300, 1000, and 3000 μM) of a mixture of three resin acids (IpA, DhA, and AbA). These concentrations are between 1 and 30 times higher than the resin acid concentrations observed in the SBR. Subsamples were taken throughout the time course, and then quantified for resin acids using GC analysis. The removal rates for all three resin acids were calculated based on these raw data. Resin acid concentrations of up to 3000 μM (0.9 mg/l) had no inhibitory effect on the ability of the biomass to remove IpA and DhA (Table 1), as indicated by the removal rates not decreasing as resin acid concentrations were increased. Interestingly, DhA removal rates continued to increase at the higher concentrations, while IpA and AbA removal rates remained constant. The control removal rates were four to ten times slower than

those with biomass. This indicated minimal abiotic removal occurred. Some of this removal was likely due to non-representative subsampling, which resulted from the resin acids sorbing to the glass flasks. Although AbA removal rates did not appear to decrease at high resin acid concentrations, no conclusion can be made about inhibition of AbA removal because of high AbA removal in the abiotic control. In summary, resin acid concentrations of up to 3000 µM did not have an inhibitory effect on resin acid removal by the SBR biomass.

Table 1: The Effect of Resin Acid Concentration on Resin Acid Removal Rates by SBR Biomass.

Treatment	Total Resin Acid Concn (µM)[b]	Resin Acid Removal Rates; µmol/L x h, (mean ± SD, n=3)[a]		
		IpA	DhA	AbA
With Biomass	100	ND[c]	12.3 ± 0.6	ND[c]
	300	18.8 ± 0.0	18.8 ± 0.0	ND[c]
	1000	31.2 ± 0.0	43.5 ± 0.0	31.2 ± 0.0
	3000	29.3 ± 2.5	59.7 ± 2.0	31.0 ± 0.2
Control Without Biomass[d]	100	2.4	2.6	ND[c]
	300	5.5	6.0	ND[c]
	1000	2.4	7.5	9.2
	3000	3.9	13.4	28.0

[a] Resin acid removal rates were calculated by subtracting the final resin acid concentration from the initial resin acid concentration, and then dividing by the hours taken to remove the resin acid.
[b] Resting cell assays containing a resin acid concentration of 100 µM were incubated for 4 h, 300µM for 8 h, 1000µM and 3000 µM for 24 h.
[c] ND, Resin acid removal rates could not be determined because removal was not linear.
[d] Uninoculated (n=1).

1.B Effect of rapid pH changes on the biomass of the SBR.

To determine if rapid changes in pH affected the ability of the biomass to remove resin acids from the SBR, biomass resting cell assays were set up at five pH values (5, 6, 7, 8, and 9) using a 100 μM mixture of three resin acids. Subsamples taken after 12 h were quantified for resin acids, and the net percent removal of each resin acid was calculated. A pH flux to 6 or 8 had no detectable adverse effect on the resin acid removal ability of the biomass (Table 2), as indicated by the complete removal of all three resin acids within the 12-hour test period. In contrast, pH spikes of 5 and 9 inhibited the ability of the biomass to remove resin acids. pH 5 had a greater inhibitory effect, compared to that of pH 9. Although the AbA data for pH 9 is not shown, the presence of over half the initial AbA at the end of the experiment indicated pH 9 partially inhibited AbA removal. All the controls, except AbA at pH 9, stayed within 90% of the initial concentration indicating abiotic resin acid removal was small. In summary, if the SBR was exposed to fluxes of pH between 6 and 8, resin acid removal was not measurably affected, but if the pH fluxed down to 5 or up to 9 the removal rates decreased greatly.

Table 2: The Effect of pH on Resin Acid Removal by SBR Biomass.

pH of Biomass	Net Percent Removal of Resin Acid (%); mean ± SD, n=3[a]		
Initial (Final)[b]	IpA	DhA	AbA
5.0 (5.0)	11.3 ± 05.2	-2.9 ± 10.2	42.4 ± 11.5
6.0 (5.8)	100.0 ± 00.0	100.0 ± 00.0	100.0 ± 00.0
7.0 (7.4)	100.0 ± 00.0	100.0 ± 00.0	100.0 ± 00.0
8.0 (8.2)	100.0 ± 00.0	100.0 ± 00.0	100.0 ± 00.0
9.0 (8.8)	33.4 ± 00.7	56.4 ± 01.2	ND[c]

[a] Net percent removal of resin acid was calculated by subtracting the final biomass resin acid concentration from the final control resin acid concentration, then dividing by the final control concentration and multiplying that value by 100.
[b] The value in ( ) represented the final pH of the experiment after a 12 h incubation
[c] ND, No value could be determined because the control went to zero.

1.C Temperature effect on numbers of and specificity of resin acid degraders in the SBR.

To determine how increased operational temperatures of the SBR affected the number and specificity of resin acid-degrading organisms in the SBR, the resin acid-degrading populations were enumerated using the most probable number (MPN) method. Tubes for MPN determination were inoculated with samples from the SBR operated at four temperatures (30°, 40°, 45°, or 50°C), and incubated at the same temperatures. At 30° and 40°C, a substantial population (10^7 to 10^8/100 ml) existed that degraded all three resin acids (Fig. 4). This population decreased ton to 10^3 degraders per 100 ml when the SBR was operated at 45°C and 50°C.

Although the data showed relatively large resin acid-degrading populations at 30°C and 40°C (Fig. 4), minor differences existed between the populations using each resin acid. For the AbA and DhA-degrading populations, the maximum population occurred at 40°C, and was ten-fold higher than the population at 30°C. The opposite was observed for the IpA-degrading population, which had a 30°C population that was greater or equal to the population at 40°C. When the IpA-degrading population was enumerated only in the presence of IpA, it increased a hundred-fold. Since, the tests were set up at different times, the increase was most likely due to an overall increase in the SBR population between sampling times, rather than the IpA-degrading population being repressed by other resin acid degraders.

At temperatures higher than 40°C, a change in the resin acid-degrading population occurred. At temperatures of 45°C and 50°C, only 10^3 degraders per 100 ml existed that could degrade any of the three resin acids as sole organic substrates (Fig. 4). When evaporator condensate was provided as another energy source, a hundred-fold higher population of DhA and IpA degraders was detected at both temperatures. These results suggest that at higher temperatures, the resin acid-degrading population requires an additional energy source to grow.

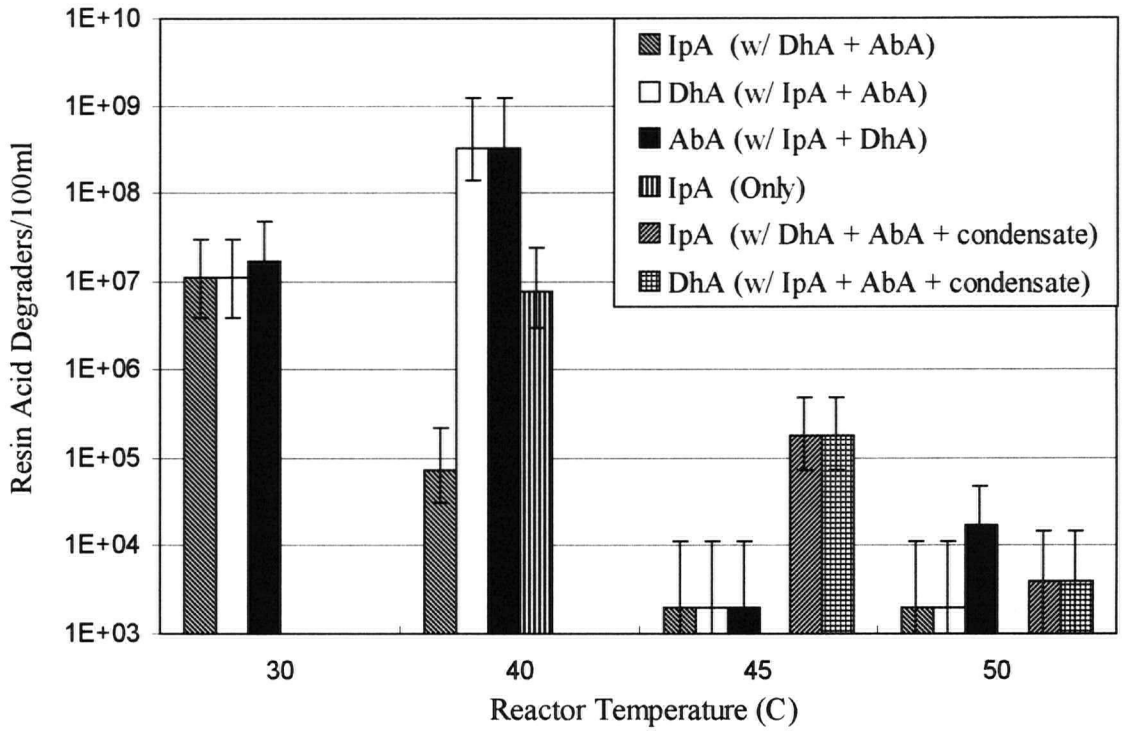


Figure 4: Resin acid-degrading populations in a sequencing batch reactor operated at different temperatures and determined by the most probable number (MPN) method.

The population values were not falsely elevated due to abiotic degradation, because no abiotic degradation occurred in any of the uninoculated controls.

To summarize the first section, fluxes in resin acid concentrations to 3000 μM (Table 1), and pH changes between 6 and 8 (Table 2), had no adverse effect on the ability of the SBR biomass to remove resin acids. At operational temperatures of 40°C and below, the SBR had a substantial resin acid-degrading population, but this population dropped to just a 10^3 degraders per 100 ml at temperatures of 45°C and higher (Fig. 4). Although these data provide information about the population of resin acid-degrading organisms and the optimal reactor conditions for the resin acid-degrading biomass, the data provided no information about the physiology of individual resin acid-degrading organisms.

2. Enrichment and isolation of resin acid-degrading organism from the SBR.

To better understand the physiology of the resin acid-degrading organisms and the biochemistry of resin acid biodegradation, attempts were made to isolate resin acid-degrading organisms from the SBR at the five operational temperatures. Two different enrichment and isolation techniques were used. The endpoint dilution method was used to isolate the most numerous resin acid-degrading organisms, while the selective enrichment method was used to isolate the fastest growing resin acid-degrading organisms. Using either method with either IpA, DhA or AbA as the sole organic carbon source and electron donor, forty-two bacterial isolates were obtained from the SBR (Table 3). Isolates were only obtained from the SBR at 20°, 30° and 40°C. Interestingly, all the 30°C strains were isolated by the end-point dilution method, whereas the 40°C strains were isolated by both methods. For the 20°C isolates only the selective enrichment method was used. At the lower two temperatures, strains were isolated on all three resin acids, whereas at 40°C no strains were isolated on DhA.

Table 3: Distribution of Bacterial Isolates Based on the Resin Acid and Temperature Used for Isolation.

Isolation Temperature (°C)	Number of Isolates on the Different Resin Acids Used for Isolation			
	IpA	DhA	AbA	Total
(RT)[a]	7[b]	6[b]	5[b]	18
30	2[c]	2[c]	2[c]	6
40	17[b,c]	0	1[c]	18
45	0	0	0	0
50	0	0	0	0
Total	26	8	8	42

[a] RT; Room temperature ranged from 19°C to 24°C.
[b] Isolates obtained by the selective enrichment method.
[c] Isolates obtained by the end-point dilution method.

2.A Screening the forty-two SBR isolates.

Using the criteria of cell morphology, colony morphology, substrate oxidation on biolog plates, substrate range and temperature range, eleven different strains emerged from the original forty-two isolates. Both gram-negative and gram-positive bacteria were represented among the eleven strains. The number of strains was reduced from eleven to five because the sole representatives of six strains lost their resin acid-degrading ability during storage at -20°C. An attempt was made to determine if this loss of degradation ability was due to plasmid loss, but no conclusive results were found. Although some strains were lost, the remaining five strains were

isolated from the SBR operated at two different temperatures (20° and 40°C). Also they were still isolated on both abietanes and pimaranes. Three of the strains were isolated on IpA (IpA-1, IpA-2 and IpA-13), while two of the strains were isolated on AbA (AbA-1 and AbA-5). The IpA-degrading strains are the first reported isolates capable of growing on IpA as a sole organic substrate. Four of the five strains represented isolates from the SBR operated at both 20°C and 30°C (AbA-1, AbA-5, IpA-1 and IpA-2), while one strain represented isolates from the SBR operated at 40°C (IpA-13). These five strains represented the following number of isolates: ten isolates were similar to AbA-1; two isolates were similar to AbA-5; seven isolates were similar to IpA-1; two isolates were similar to IpA-2; and 6 isolates were similar to IpA-13.

As mentioned previously, colony morphology also differentiated the bacteria into strains. When grown on AbA, colonies of AbA-1 were yellow, opaque, smooth, convex, shiny and symmetrical. When grown on IpA, colonies of AbA-5 and IpA-1 were clear, translucent, smooth, flat, and asymmetrical. Colonies of IpA-2 were pale yellow, opaque, smooth, convex, and round, and colonies of IpA-13 were white, opaque, fuzzy-rough, flat, and round. Physiological characteristics were also used to distinguish the strains (Table 4).

2.B Physiological and biochemical characterization of the five SBR strains.

To determine their physiological and biochemical characteristics, the five strains were put through a standard battery of biochemical tests. This physiological characterization included Biolog characterization, growth at 6 temperatures and growth on thirty-two substrates, which included many carbon sources likely present in the SBR. All five resin acid-degrading bacteria had similar trends in their overall use of substrates (Table 4). This was exemplified by the strains' use of nearly all resin and fatty acids tested, which included the major resin and fatty acids of the

Table 4: Characterization of Five Resin Acid-Degrading Strains Isolated from the SBR.

	Substrate Concn (g/liter)	Physiological Reaction or Use of Substrate[a]				
		AbA-1	AbA-5	IpA-1	IpA-2	IpA-13
Gram	--	-	-	-	-	+
Shape	--	Rod	Rod	Rod	Rod	Rod
Motility	--	+/-	-	+	+	-
Clumping	--	-	-	-	-	+
Oxidase	--	-	+	+	+	-
Catalase	--	-	+	+	+	+
Max. Growth Temperature	--	30° C	37°C	37°C	30°C	40°C
Use of Resin Acids:[b]						
Isopimaric	0.06	NG	G	G	G	G
Pimaric	0.06	NG	G	G	G	G
Dehydroabietic	0.06	G	(G)	(G)	(G)	G
Abietic	0.06	G	G	(G)	NG	G
12, 14-Chloro-DhA[c]	0.12	G	G	NG	NG	NG
Use of Fatty Acids:						
Linoleic acid	1.0	NG	G	G	G	NG
Palmitic acid	1.0	G	G	G	G	G
Use of Wood Sugars:						
L-Arabinose	1.0	G	(G)	(G)	G	NG
Carboxymethyl-cellulose	1.0	(G)	NG	NG	NG	NG
Cellobiose		O	O	NO	NO	NO
D-Galactose	1.0	G	NG	NG	G	NG
D-Glucose	1.0	NG	G	G	G	NG
Glucuronate		O	NO	NO	O	NT
D-Mannose	1.0	NG	NG	NG	G	(G)
Xylan	1.0	NG	NG	NG	NG	NG
D-Xylose	1.0	G	NG	NG	(G)	NG
Use of Other Substrates:						
Acetate	1.0	G	G	G	G	G
Betulin[b]	0.1	NG	NG	NG	NG	G
Camphor[b]	0.1	NG	NG	NG	NG	NG
β-Citronellol[b]	0.1	NG	G	G	NG	NG
Ethanol	0.2	G	G	G	G	NG
Glycerol	1.0	G	(G)	G	G	NG
Hexadecane[b]	0.5	NG	G	G	NG	NG
Methanol	0.1	NG	NG	NG	NG	NG
Pyruvate	1.0	G	(G)	(G)	G	(G)
β- Sitosterol[b]	0.1	G	NG	NG	NG	G
Use of Aromatic Compounds:[b]						
Benzoate	0.24	G	G	G	G	G
3-Chlorobenzoate	0.20	NG	NG	NG	NG	NG
Biphenyl	0.20	NG	NG	NG	NG	NG
Napthalene	0.20	NG	NG	NG	NG	NG
Phenol	0.01	NG	NG	NG	G	NG
Pentachlorophenol	0.01	NG	NG	NG	NG	NG

[a] **G** supports growth as sole organic substrate; **(G)** Supports poor growth; **NG** Does not support growth; **O** Oxidized in Biolog assay; **NO** Not oxidized; **NT** Not tested.

[b] For these substrates, growth is defined as ability to grow and completely remove substrate in at least three serial cultures. Cultures were incubated on a shaker or a tube-roller.

[c] 12,14-Chloro-DhA exists as a mixture of 44% 12-Cl-DhA and 48% 14-Cl-DhA.

SBR influent. This was also indicated by their limited use of the non-resin and fatty acids substrates tested. For example, any one individual isolate used only a maximum of 50 percent of the other substrates. Of the other substrates tested, all five strains used a few, but generally different, wood sugars. They all used possible fermentation byproducts, such as acetate or ethanol, which could have been produced during the non-aerated settling phase of the SBR. Of the aromatic compounds tested, the isolates, with the exception of IpA-2, only used benzoate, and strain IpA-2 also used phenol. Interestingly, incubation of strains AbA-1, AbA-5, IpA-1 and IpA-2 with 3-CB turned the medium a black color, suggesting a 3-chlorocatechol degradation product [33]. Only AbA-5 and IpA-1 grew anaerobically on glucose when nitrate served as an electron acceptor, whereas none of the strains fermented glucose. IpA-13 neither fermented pyruvate or grew on pyruvate anaerobically when nitrate served as an electron acceptor. In addition to using nitrate as an electron acceptor, AbA-5 and IpA-1 used nearly identical substrates.

Although individual isolates were relatively similar in their overall trends of substrate use, the isolates differed based on three parameters. The strains can be subdivided into two broad groups based on use of resin acids. AbA-1 grew on AbA, DhA and Cl-DhA, and therefore is an abietane degrader (Table 4). The other four strains, which grew on IpA and PiA, are pimarane degraders. The use of resin acids will be discussed in more depth in the next section. A second difference was between the gram-negative strains and the one gram-positive strain represented by IpA-13. All the gram-negative strains readily used some of the non-resin and fatty acid substrates tested, but IpA-13 only used limited substrates (33%) including resin and fatty acids. IpA-13 also used the plant compounds betulin (a triterpene) and β-sitosterol (a steroid). Citronellol, another plant compound, was not used by IpA-13, but was used by AbA-5 and IpA-1. Lastly, using the maximum growth temperature, isolates can be divided into the following three groups: ones that

only grew up to 30°C (AbA-1 and IpA-2); ones that grew up to 37°C (AbA-5 and IpA-1); and lastly those that grew up to 40°C (IpA-13).

2.C Growth on resin acids.

Since growth on resin acids was one of the major differentiating characteristics, the use of resin acids by all five strains is discussed in more detail. Strains were considered positive for resin acid use if they grew on and completely removed the resin acid in three consecutive transfers. AbA-1, an abietane degrader, used only abietanes, such as AbA and DhA (Table 4). AbA-1, also grew on and completely removed a Cl-DhA mixture (12-Cl-DhA and 14-Cl-DhA). The individual Cl-DhAs could not be tested, as they were not available as single compounds. The other four pimarane degraders used the pimaranes, IpA and PiA, and additionally grew on the two abietanes, except for IpA-2 which only grew on DhA. In addition, AbA-5 grew on and completely removed the Cl-DhA mixture. On the other hand, IpA-1 which is identical to AbA-5 in most other ways did not grow on the Cl-DhA mixture. When both AbA-1 and AbA-5 were grown on the Cl-DhA mixture, one apparent metabolite was consistently produced. The metabolite had a relative retention time of 0.770 compared to that of the internal standard. Although the four pimarane degraders grew on the abietanes, three of the four strains grew faster and to higher density on the pimaranes. IpA-13 was the only strain that seemed to use all the resin acids equally. All four pimarane degraders grew well on PiA, yet PiA often was not completely degraded by cultures even after long (14 d) incubations.

3. The IpA-degrading isolates of the SBR.

The rest of my research focused on the pimarane degraders because these are the first isolates reported to be capable of growth on IpA (Fig. 1) as the sole carbon and energy substrate

(Table 4). This decision was supported by the similarity of the abietane-degrading strain, AbA-1, to a previously published resin acid-degrading strain, DhA-33 [32]. Of the four strains, three were chosen to represent different IpA degraders in the SBR. The fourth isolate, AbA-5, was not further investigated because it turned out to be nearly identical to another strain, IpA-1. Both IpA-1 and IpA-2 were isolated from the SBR at 20°C, but are considered isolates of the SBR at both the 20°C and 30°C. This is because strains similar to both were also isolated from the SBR at 30°C. IpA-13 is considered to be an isolate of the SBR at 40°C.

3.A Kinetics of growth on IpA.

Growth curves and IpA removal curves were measured for the three IpA-degrading strains to investigate whether growth was correlated to IpA removal. The experiments were set up in individual tubes, which contained 1 ml of culture medium supplemented with IpA to a final concentration of 100 μM. Individual samples were used for both protein and IpA analyses because the entire culture was required for the assay, due to the difficulty in obtaining representative subsamples. Samples were frozen at each time point and quantified for IpA and protein. Growth of IpA-1 and IpA-2 on IpA correlated to IpA removal, as indicated by the proportional increase in protein and the simultaneous decrease in IpA (Figs. 5A and 5B). Results from the uninoculated control cultures indicated that abiotic removal of IpA had not occurred.

For IpA-13, the correlation between growth and removal seemed more questionable (Fig. 5C). The IpA-13 results should be viewed only as a trend because the filtration collection method used for the protein assay resulted in high variability. However, IpA was completely removed. No abiotic removal occurred in the uninoculated controls indicating IpA removal was associated with the growth of IpA-13. During the growth curves for IpA-13 microscopy revealed two phases of growth with distinctive morphologies associated with each phase. IpA-13 started

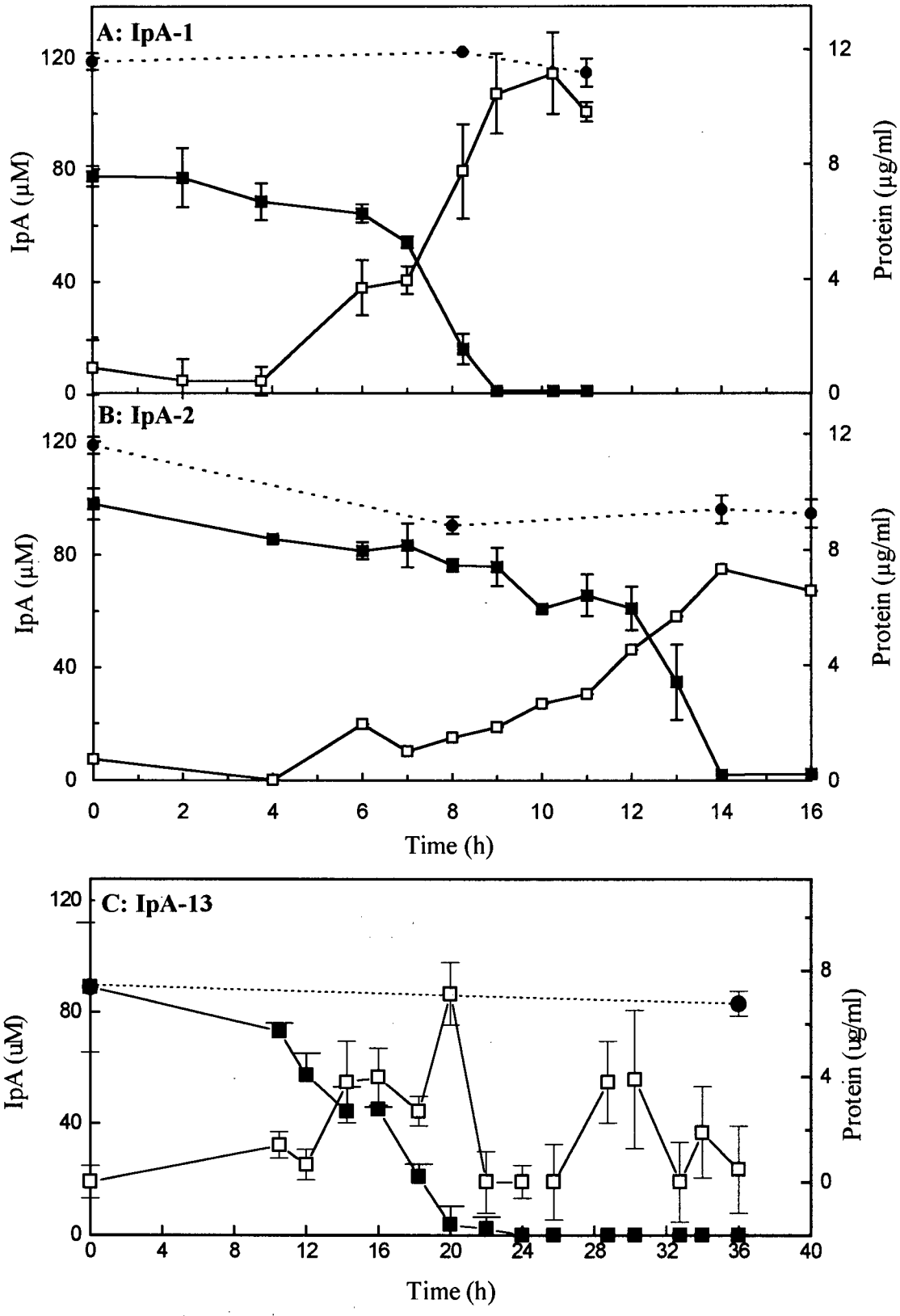


Figure 5: Growth on and removal of 100 µM IpA by strains IpA-1 at 30oC (A), IpA-2 at 30oC (B) and IpA-13 at 40oC (C); ■, IpA; □, Protein; ● , Uninnoculated control IpA; bars indicate standard deviation (n=3).

out as short rods (Fig. 6A) and then the rods elongate (Fig. 6B). While IpA was being removed, large mats of rods developed (Fig. 6C). During stationary phase, when no IpA was present, the large mats of long rods were converted back to short rods (Fig. 6D), which broke apart and resembled the initial culture (Fig. 6A).

Strains IpA-1 and IpA-2 are more similar to each other in their growth on and removal of IpA at 30°C, than to IpA-13 at 40°C (Fig. 5). The lag phases of IpA-1 and IpA-2 were three to six hours shorter than the lag phase of IpA-13. The primary growth and IpA removal phases of IpA-1 and IpA-2 also occurred in half the time of IpA-13. In addition, growth of strains IpA-1 and IpA-2 on IpA were not exponential and occurred at maximum rates of 3 and 4 μg of protein ml^{-1} h^{-1}, respectively. Lack of exponential growth was attributed to a consistently observed pause in growth which occurred 2 to 3 hours into the growth phase for both organisms. This pause always occurred before the majority of the IpA was removed. Because of a highly-variable growth curve, the growth rate of IpA-13 was not calculated. Growth rates for all three strains may have been limited by the insolubility of IpA.

3.B Growth on IpA at different temperatures.

Growth curves and IpA removal curves were also determined, as described earlier, to investigate how an organism in the SBR would compete at the different operational temperatures of the SBR. The regular occurrence of a pause in both growth and IpA removal for the IpA-degrading strain prevented the calculation of comparable growth rates between these strains. Therefore, growth curves and IpA removal curves of IpA-2 at three temperatures (20°, 25° and 30°C) and at one or both initial IpA concentrations (100 μM and 200μM) were examined as preliminary indications of competition within the SBR. Using the growth period as an indicator,

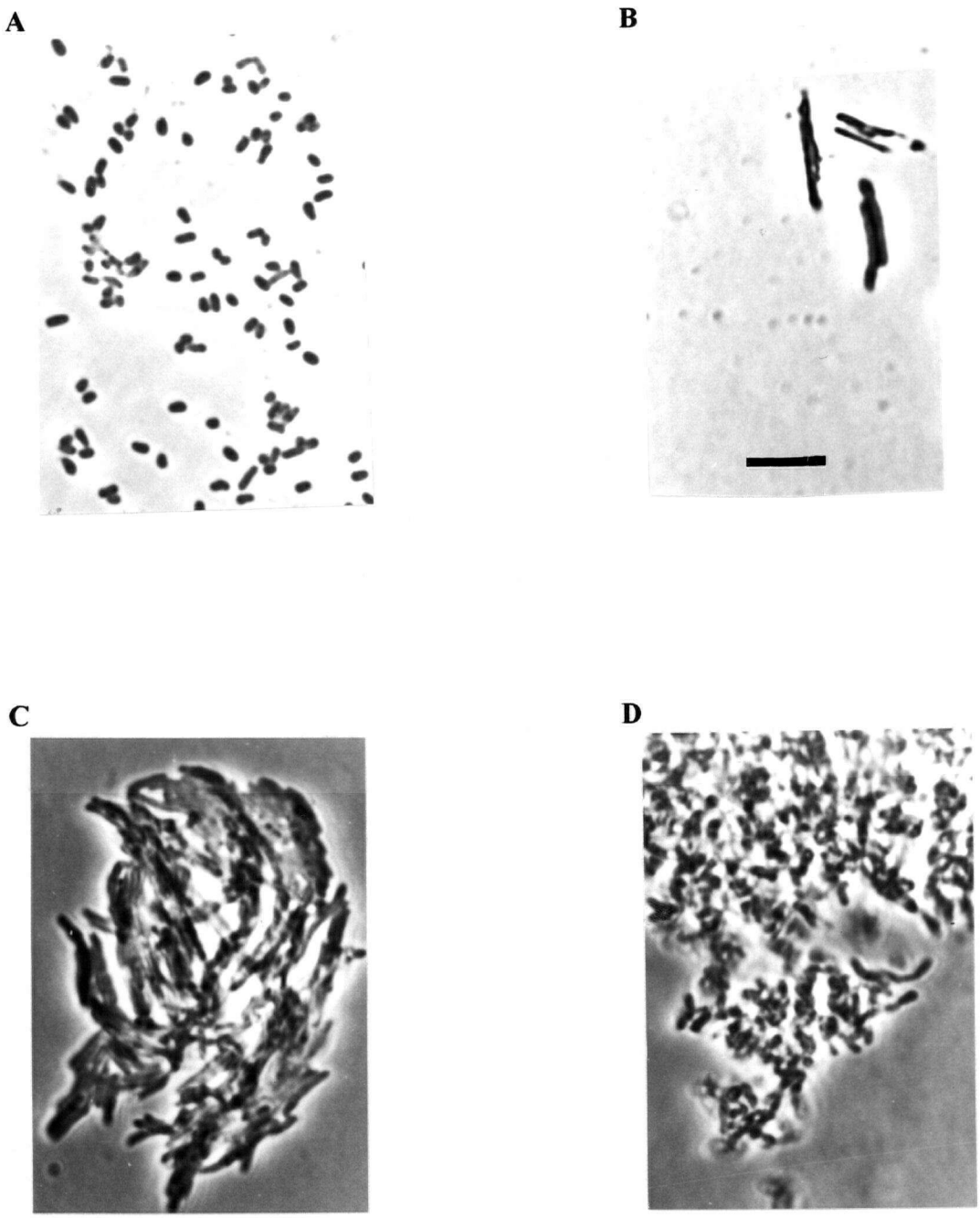


Figure 6: Photomicrographs of strain IpA-13 at different growth phases and different cell morphologies during growth on 0.06 g/l IpA; inoculum (A); elongation of rods (B); rods aggregated to a large mat (C); stationary phase cells are converting back to short rods (D); Bar, 5.0 µm.

initial IpA concentrations rather than growth temperatures affected the growth of IpA-2 (Figs. 5B, 7A, 7B and 7C). This was completely due to IpA-2 having roughly half the lag phase period if grown on an initial IpA concentration of 100 μM, compared to growth on an initial concentration of 200 μM. After different lag periods, the period of growth and IpA removal was the same for both concentrations. When the amount of protein produced was used as an indicator of competitiveness, temperature, not initial IpA concentration primarily affected growth of IpA-2, as shown by the two to four-fold higher protein concentrations for IpA-2 at 20°C, compared to values at either 25°C or 30°C. Interestingly, different concentrations of IpA produced the same concentration of protein at the lower temperatures. One explanation for this might be that more of the IpA irreversibly bound to the glass and therefore was unavailable, when the IpA concentration was higher. This was supported by the partial disappearance of IpA in both the uninoculated controls and the early time points of the growth curves at 20°C.

Maximum specific IpA degradation activity was another way to investigate the IpA-removal potential of different strains within the SBR at the different operational temperatures. The growth curve data (Figs. 5 and 7) were used to calculate maximum specific IpA degrading activity values for all three strains. The maximum specific IpA degrading activity of an individual isolate was defined as the variable point during growth on IpA that IpA removal was maximal, when normalized to the μg quantity of protein. Maximum specific activity for IpA-2 was not temperature dependent, as indicated by the variable values at both low and high growth temperatures (Fig. 8). Maximum specific activity may have been strain dependent. IpA-2 had twenty percent more specific activity than that of IpA-1 at 30°C. IpA-13, grown at 40°C, had the highest maximum specific activity, even though the protein yields for IpA-13 were equal to those of IpA-2 at 30°C.

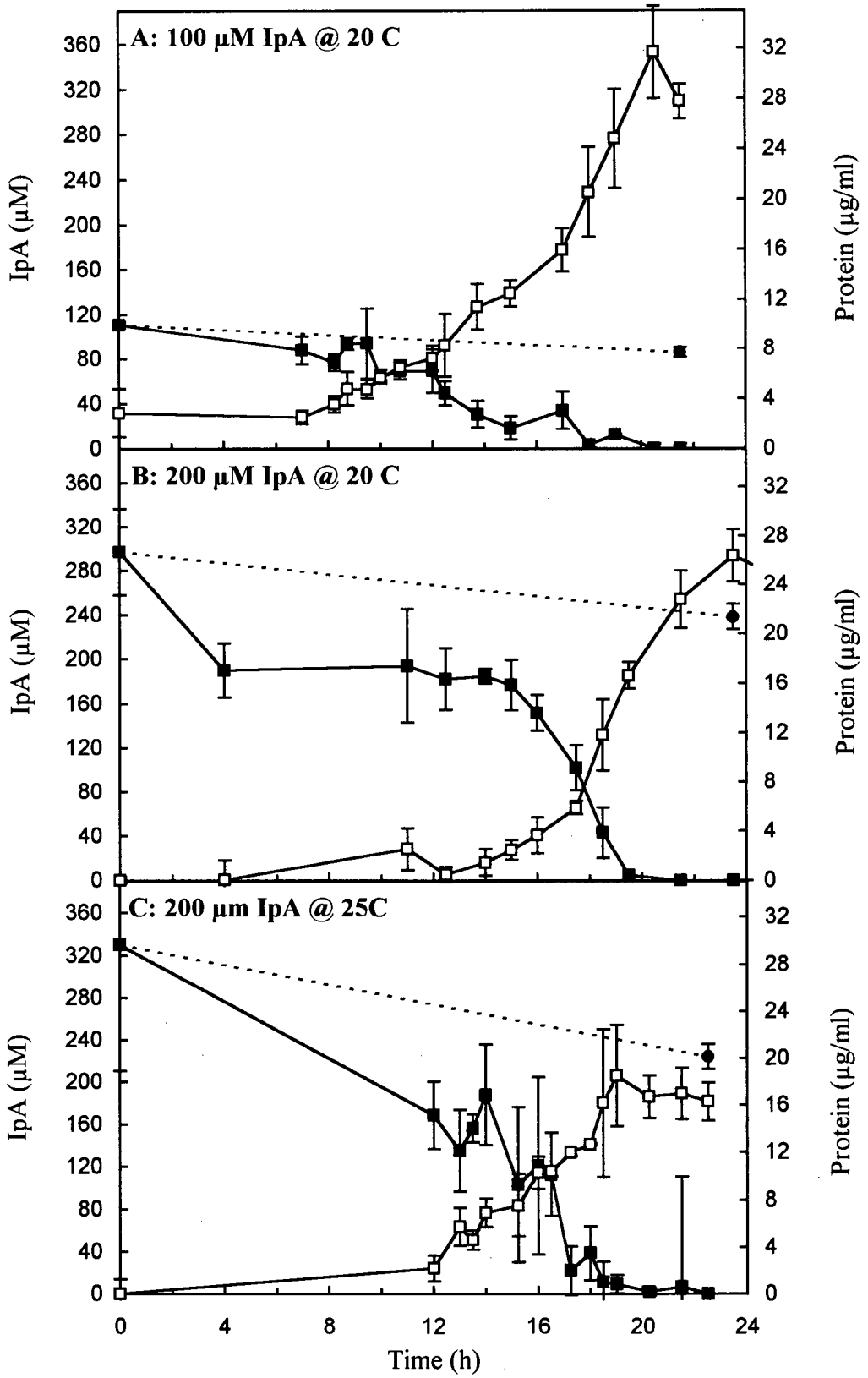


Figure 7: The effect of temperature and IpA concentration on the growth and removal of IpA by strain IpA-2 at 20°C on 100 µM IpA (A), at 20°C on 200 µM IpA (B) at 25°C on 200 µM IpA (C); ■, IpA; □, Protein;● , Uninnoculated control IpA; bars indicate standard deviation (n=3).

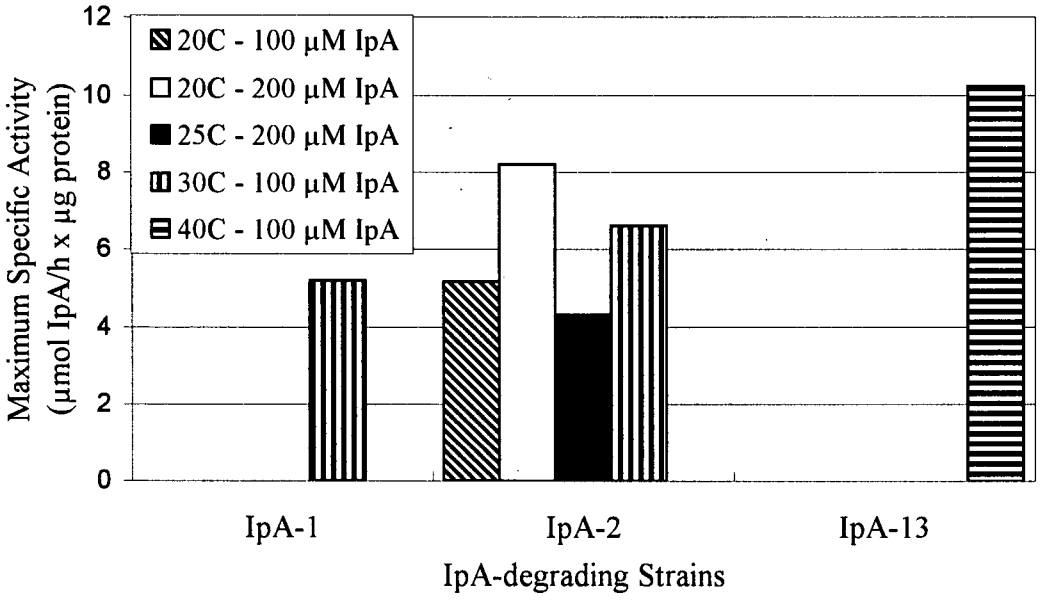


Figure 8: Maximum specific IpA degradation activity of IpA-degrading strains growing at different temperatures with different initial IpA concentrations. Maximum specific IpA degradation activity was calculated from the previous IpA removal curve and IpA growth curve data (Figs. 5 and 7).

3.C Growth yields on IpA.

Growth on IpA was previously shown to be correlated to IpA removal (Fig. 5), but mineralization of the IpA was not established. One way of estimating whether complete mineralization of IpA occurred was to measure the amount of protein produced from a given amount of IpA. Theoretically, when a substrate is mineralized under aerobic conditions, approximately one half of the substrate is converted to biomass. Since roughly one half of the cell biomass is protein, roughly one-quarter the mass of the substrate can be measured as protein. To determine if IpA-1, IpA-2 and IpA-13 mineralized IpA, cultures containing different amounts of IpA (0.0, 0.73, 1.46, and 2.19 mg) were grown to early stationary phase and then assayed for protein. Optical density was also measured for cultures of IpA-1 and IpA-2, but not for IpA-13. IpA-1 and IpA-2 showed a linear relationship between cell yield (as protein or optical density) and IpA provided (Figs. 9A and 9B). This indicated that IpA was the limiting growth factor in the cultures. Also, when mg of IpA was converted to μM of IpA, IpA was shown not to be toxic at a concentration of 600 μM (0.18 g/l). However, a linear relationship was not demonstrated for IpA-13 (Fig. 9C) Two explanations for this can be given, either IpA was toxic to IpA-13 at concentrations above 200 μM or, since all the IpA was not consumed, the cell yield values are not truly representative. To estimate if complete mineralization had occurred, the protein values for IpA-1, IpA-2 and IpA-13 (Fig. 9) were converted to the growth yield values of 0.19, 0.23, and 0.27 g protein per g IpA, respectively. Assuming the cells were 55% protein, these growth yields corresponded to 0.35, 0.42, and 0.49 g of cell dry weight per g of IpA, respectively. No indication can be made about complete mineralization of IpA by these strains because these values are lower than the expected theoretical yield of 0.50 g of cell dry weight per g of IpA for two of the three strains.

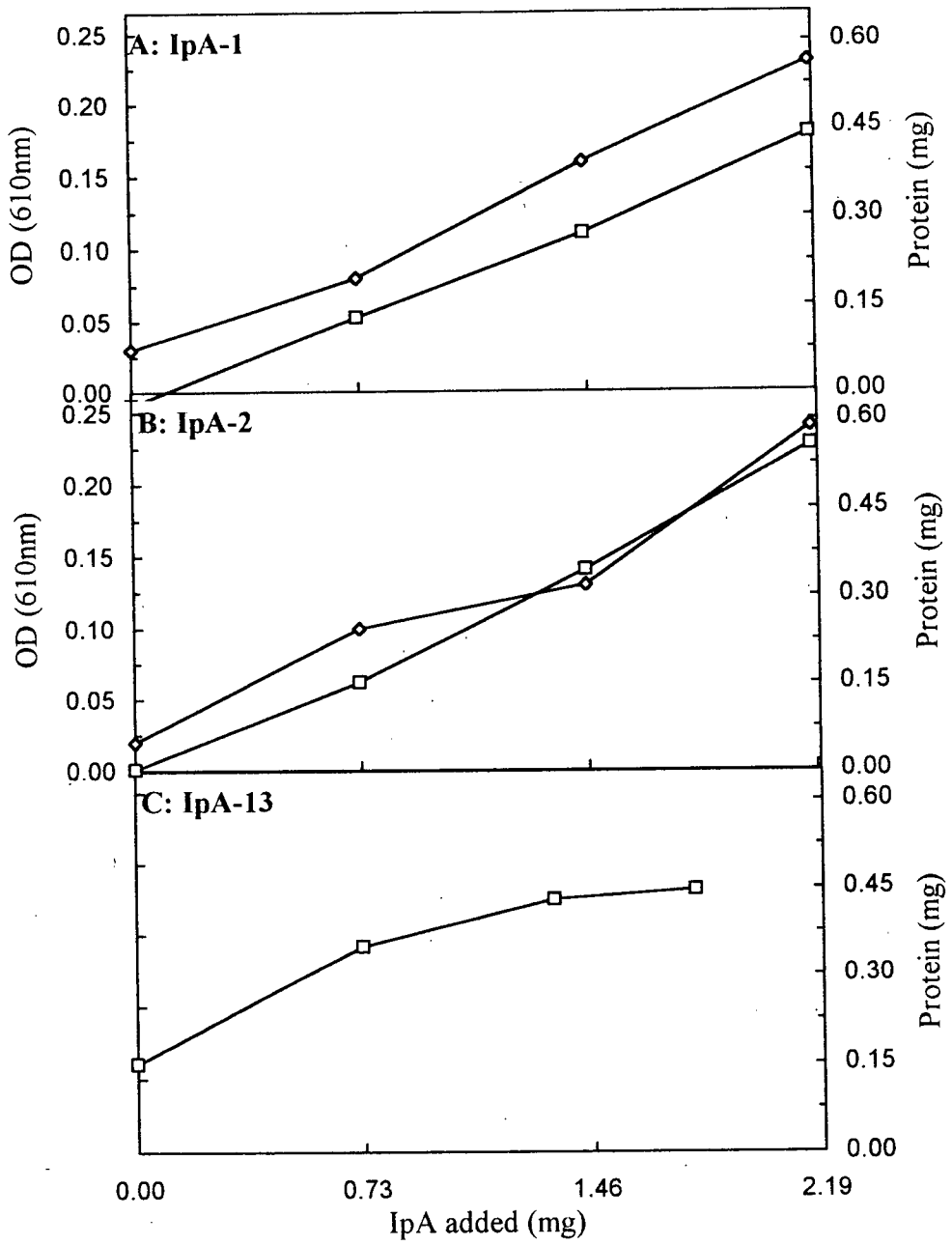


Figure 9: Growth yields of IpA-1 at 30°C (A), IpA-2 at 30°C (B), and IpA-13 at 40°C (C) on IpA; □ , protein; ◇ , OD. Datapoints are the average of duplicate samples for IpA-1 and IpA-2, and triplicate samples for IpA-13.

3.D. Carbon mass balance determinations for IpA degradation.

Because the growth yields were lower than expected more quantitative, carbon mass balance experiments were done to conclusively determine if IpA-1, IpA-2, and IpA-13 completely mineralized IpA. For all three strains, the fate of carbon during growth on IpA was determined using individual early stationary phase cultures. The cultures were assayed for either IpA, CO_2, or protein and dissolved organic carbon (DOC). The values were converted to percent of total initial carbon (Fig. 10). When previously grown on IpA, all three isolates converted approximately one-third of the carbon to biomass, one-third, to CO_2, and one-third, to DOC. No IpA remained in any of the cultures. The biomass and CO_2 values were again below the expected 50% theoretical conversion to conclusively confirm IpA was completely mineralized. Although complete mineralization of IpA was not confirmed, all three strains partially mineralized two-thirds of the initial carbon to biomass and CO_2. One explanation for these lower than expected values could be low carbon recovery, but 100% to 105% for IpA-1 and IpA-2, and 112% for IpA-13 of the carbon was accounted for. To ensure IpA was not being abiotically converted to other forms of carbon, uninoculated controls were examined. These had recoveries of 80% for IpA-1 and IpA-2, and 68% for IpA-13. Irreversible binding of IpA to the rubber stoppers may explain this loss of IpA.

Because one-third of the carbon was converted to DOC by all three strains (Fig. 10), attempts were made to identify this component. First, the molecular weights of the DOC were roughly determined using molecular cutoff filters. Mixed results were observed for IpA-1 and IpA-2. For IpA-1, the majority of the DOC had a molecular weight greater than 10 kD, because 68% of the DOC was retained by a filter with a molecular weight cutoff of 10,000. For IpA-2, the majority of the DOC had a molecular weight less than 10 kD, because only 33% of the DOC was retained by the same size filter. Although the DOC was not identified, a few things can be

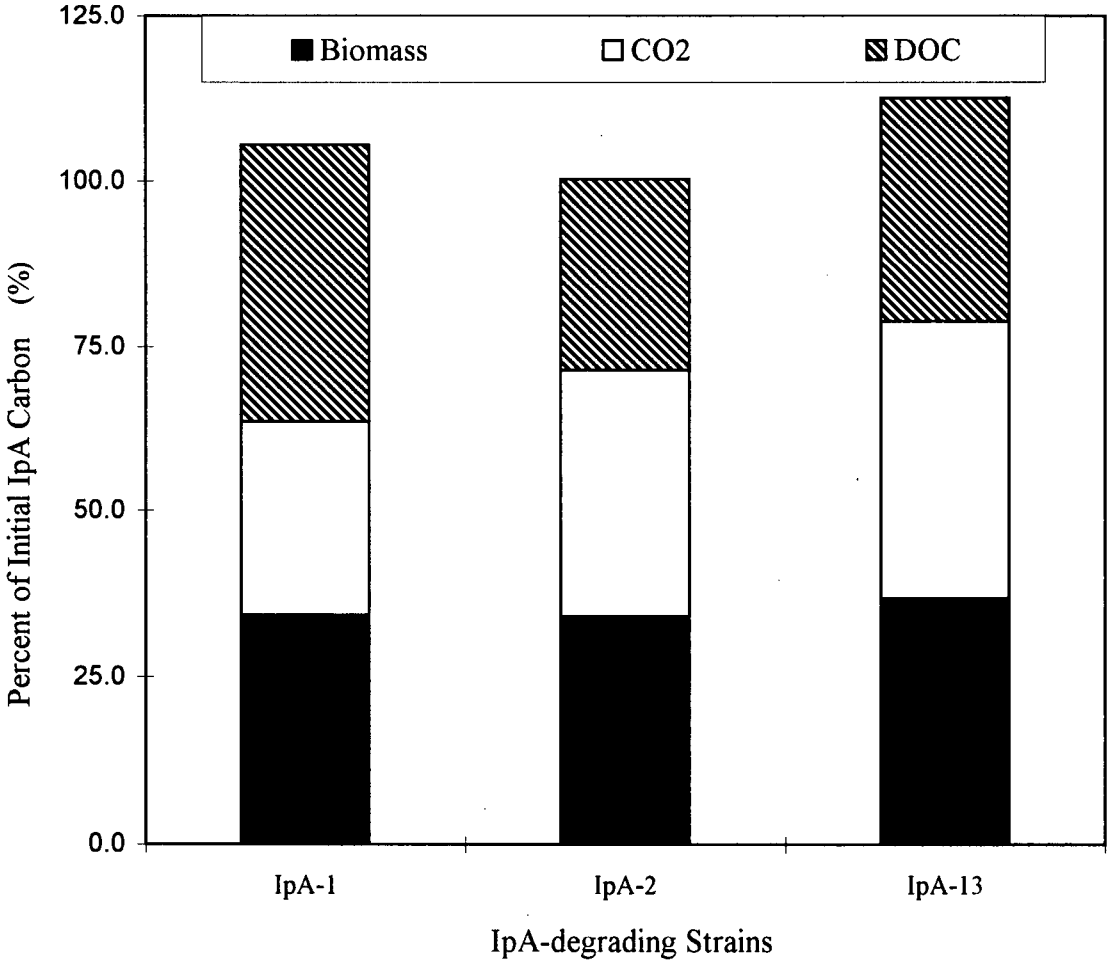


Figure 10: Carbon mass balance of IpA-1, IpA-2 and IpA-13 grown on 0.06 g/l of IpA at 30°C and 40°C, respectively; (n=3). Uninoculated control values were designated 100% IpA recovery; (n=3).

excluded. The DOC fraction did not contain IpA because no IpA remained in any of the cultures. The DOC fraction also did not contain compounds such as IpA degradation metabolites detectable by the GC analysis, because no additional peaks were observed when the samples were assayed for IpA. One possible identity of the DOC component would be small metabolites that were undetected during GC analysis for IpA. The DOC might also contain a secreted product or cellular components that were released. An example of a plausible cellular component or product would be a biosurfactant that is released by the IpA-degrading bacteria which may solubilize IpA.

3.E Assaying for a biosurfactant.

Several pieces of data and observations throughout my research support the hypothesis that IpA degraders may produce a biosurfactant. First, during growth on IpA, a pause was observed both in growth and in IpA removal (Figs. 5A and 5B). Second, one-third of the overall carbon was converted to DOC by all three IpA-degrading strains (Fig. 10), yet the growth yields were on average only 11% below the theoretical 25% yield. Lastly, it was observed microscopically that solubilization of IpA might occur just prior to maximum growth and IpA removal. To test the hypothesis that IpA degraders produced a biosurfactant, the dynamic surface tension of a IpA-1 culture was assessed by direct measurement of surfactant activity on individual 5-ml cultures of IpA-1 that were harvested at selected times during growth. Along with surfactant activity samples, IpA and protein samples were taken at each time point and frozen. The protein and IpA values were compared to two different measurements, which are indicators of dynamic surface tension (Figs. 11 and 12). The σ_{20} measured the surface tension change at a fixed time, as a function of pressure. The t_x measured the time, in seconds, when the surface tension initially changed at a fixed pressure. Both these measurements are inversely proportional to the concentration of a biosurfactant [36]. The surfactant activity, as shown by both

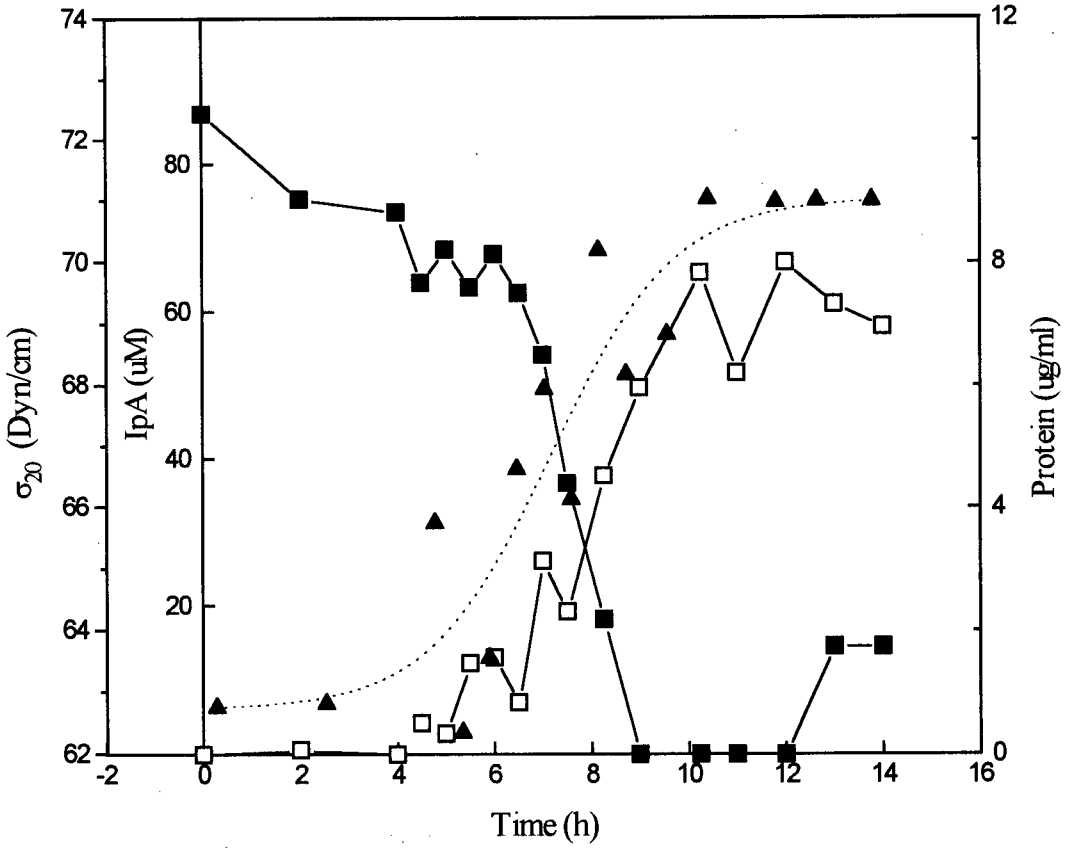


Figure 11: Biosurfactant activity of IpA-1 grown on 100 μM IpA at 30°C as measured by dynamic surface tension (σ_{20}) at a bubble age of 20 sec; ▲, σ_{20}; ■, I p A ; □, Protein; (n=1).

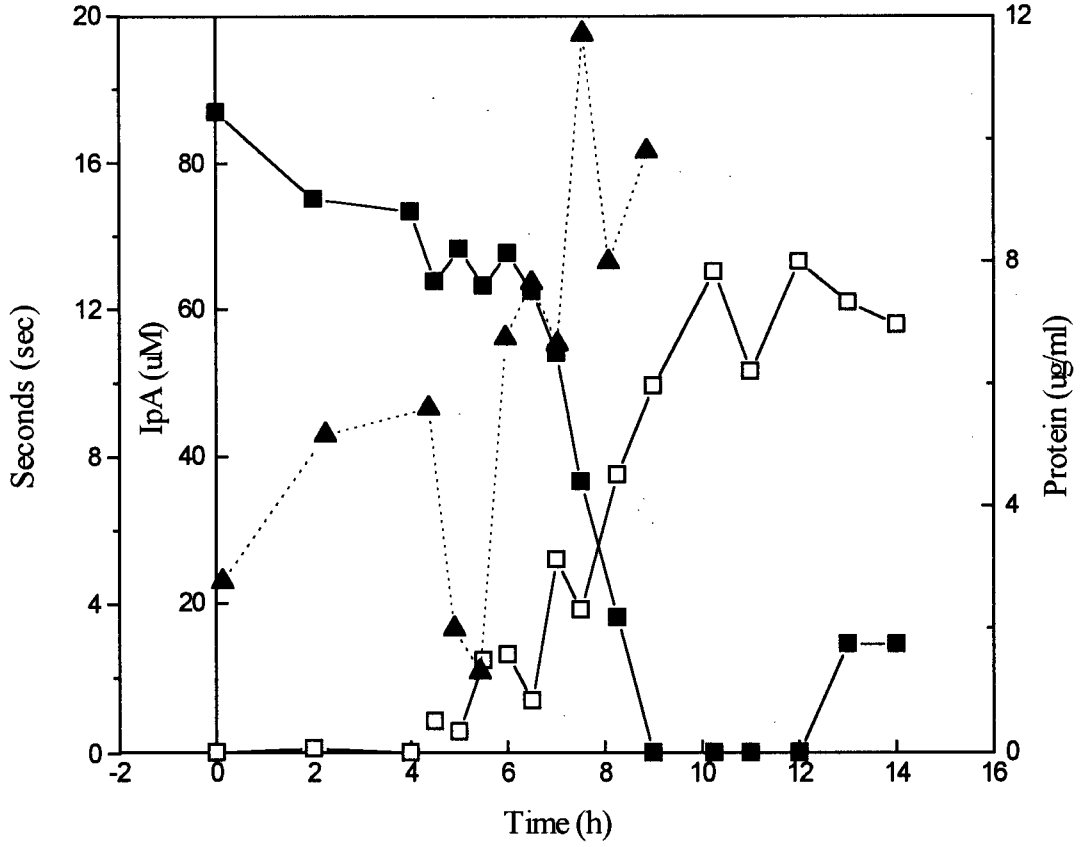


Figure 12: Biosurfactant activity of IpA-1 grown on 100 µM IpA at 30°C as measured in seconds taken for initial surface tension change; ▲ , t_x (seconds); ■, IpA; □, Protein; (n=1).

measurements, correlated with the expected surfactant activity of IpA, rather than to growth of IpA-1 (Figs. 11 and 12). At the beginning of the time course, the expected low values for both measurements indicated IpA produced surfactant activity. Following the initial low values, both values subsequently increased indicating the disappearance of surfactant activity. This disappearance occurred simultaneously with the removal of IpA by IpA-1. During growth of IpA-1, no change occurred in either of the measurements, which indicated IpA-1 most likely did not produce a surface active agent during growth. One, slight anomaly, indicated by the dramatic shortening of t_x between the fourth and fifth hour of the time course, may suggest a sudden production of a biosurfactant by IpA-1. But these are only two points on a very scattered, single replicate plot. If biosurfactant were produced, the presence of that surfactant should remain evident after that timepoint. However, no surfactant activity was present, as indicated by the measurements of surface tension being equal to that of deionized water. Therefore, σ_{20} and t_x measurements do not support the hypothesis that biosurfactant was produced by strain IpA-1, since only IpA, not growth of IpA-1 showed surfactant activity.

To summarize section 3, the IpA-degrading strains grew on IpA as a sole organic carbon and energy source. Growth on IpA was correlated with IpA removal (Figs. 5A and 5B), and this conversion resulted in growth yields between 0.19 g and 0.27 g protein per g IpA. A carbon mass balance determination revealed that only two-thirds of the carbon was converted to biomass and CO_2, while one-third remained as dissolved organic carbon (Fig. 10). One hypothesis for the identity of the DOC fraction was a biosurfactant, but testing of IpA-1 provided no evidence to support this hypothesis (Figs. 11 and 12).

4. Induction of IpA degradation.

The last focus of my research was to investigate if IpA degradation is an inducible degradation activity. IpA-1 and IpA-2 were used as representative IpA-degrading strains. Both strains were grown on either IpA or glucose, then cell suspension assays were set up with four analytical-grade resin acids (IpA, PiA, AbA, and DhA). In a preliminary trial, cell suspensions of IpA-1 and IpA-2 grown on IpA, completely removed the IpA in 4 hours and the initial IpA removal rates for both strains were linear (Fig. 13). When the IpA removal rates were compared between cell suspensions with or without chloramphenicol, the removal rates were slower in the presence of chloramphenicol, a protein synthesis inhibitor. This suggests that translation is required for degradation. Chloramphenicol would block the translation of a message that encoded an essential factor and therefore removal of new IpA would be slower, because removal would be limited to what the existing preinduced enzymes could remove. The IpA removal rates in the presence of chloramphenicol were 0.56 and 0.13 μmol mg of protein^{-1} h^{-1} for IpA-1 and IpA-2, respectively. IpA removal activity was also heat labile since boiling the cells stopped all activity. No lag phase existed for either IpA-1 or IpA-2, grown on IpA, which suggested the cells were preinduced by growth on IpA.

Using the preliminary data, a larger cell suspension experiment was set up for both strains to test the inducibility of resin acid removal. Both strains had an inducible, heat-labile IpA removal activity (Table 5). This induced removal activity was evident when a cell suspensions of IpA grown cells, in the presence of the protein synthesis inhibitor chloramphenicol, completely removed IpA. In contrast, cell suspensions of glucose-grown cells under the same conditions, did not remove IpA. This second observation indicated that IpA removal was not a constitutive activity. In addition, the removal of the other three resin acids was induced in the presence of IpA as indicated by the same removal patterns stated above. Interestingly, removal of AbA by IpA-2 may have been a co-metabolic process because IpA-2 did not grow on AbA. Surprisingly,

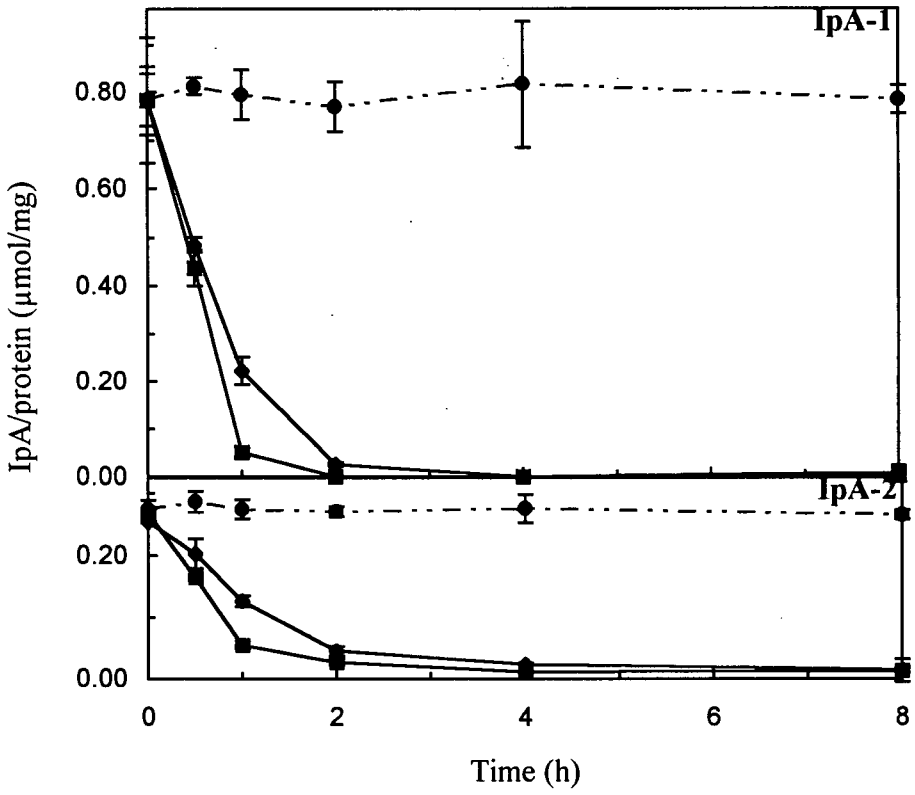


Figure 13: Removal of IpA by cell suspensions of IpA-1 and IpA-2; ■, without chloramphenicol; ◆, with chloramphenicol; ●, boiled controls; bars indicate standard deviation (n=3).

Table 5. Data from Induction Experiments Showing the Removal of Four Resin Acids by Cell Suspensions of Strains IpA-1 and IpA-2.

Strain	Growth[a] Substrate	Treatment	% removal of resin acid (mean ± SD, n=3)[b]			
			IpA	PiA	DhA	AbA
IpA-1	IpA	W/ Chloramphenicol	100 ± 0.0	87 ± 2.7	100 ± 0.0	100 ± 0.0
		W/o Chloramphenicol	100 ± 0.0	91 ± 1.4	100 ± 0.0	100 ± 0.0
		Boiled Control	15 ± 12.1	18 ± 6.4	- 9 ± 10.3	8 ± 6.7
	Glucose	W/ Chloramphenicol	- 1 ± 8.2	- 26 ± 15.6	- 3 ± 8.1	2 ± 1.4
		W/o Chloramphenicol	100 ± 0.0	8 ± 29.4	6 ± 5.3	13 ± 2.6
		Boiled Control	12 ±13.3	9 ± 19.3	- 48 ± 15.2	15 ± 6.1
IpA-2	IpA	W/ Chloramphenicol	100 ± 0.0	77 ± 3.4	100 ± 0.0	100 ± 0.0
		W/o Chloramphenicol	100 ± 0.0	93 ± 2.0	100 ± 0.0	100 ± 0.0
		Boiled Control	- 21 ± 47.2	14 ± 24.6	- 5 ± 5.0	1 ± 3.1
	Glucose	W/ Chloramphenicol	11 ± 8.1	7 ± 1.9	- 1 ± 0.9	- 5 ± 42.9
		W/o Chloramphenicol	100 ± 0.0	74 ± 2.4	80 ± 17.4.	80 ± 0.5
		Boiled Control	2 ± 5.9	7 ± 10.4	13 ± 10.9	0 ± 7.0

[a] Suspensions of cells grown on IpA (induced) were incubated for 8 h, and suspensions of cells grown on glucose (uninduced) were incubated for 10h.
[b] The initial resin acid concentration was 200µM.

without the chloramphenicol, IpA-2, grown on glucose, removal of each resin acids was initiated in the presence of that particular resin acid, but only IpA was removed in the presence of itself by IpA-1 grown on glucose. Since IpA-1 grew on all four of the resin acids, this difference between the strains was most likely due to the requirement of a longer induction period for IpA-1, rather than lack of induction. For both strains, induction of IpA removal appeared to be faster than induction of removal of the other resin acids. Lastly, no resin acid removal occurred in the boiled control suspensions, indicating that the removal activity was heat labile.

Cell suspensions and cultures of IpA-1 and IpA-2 yielded apparent metabolites from DhA and PiA. Relatively large amounts of three apparent DhA metabolites with GC retention times of 6.2, 8.9 and 9.6 min (Fig. 14) were consistently produced and were shown to accumulate. Two transient compounds were also observed when cells metabolized DhA. Both cell suspensions (Table 5) and cultures failed to completely remove PiA. A relatively small amount of one apparent PiA metabolite was consistently produced and was stable. No, IpA or AbA metabolites were detected. The patterns of apparent metabolites were identical for both IpA-1 and IpA-2. Since the retention times of the all the apparent metabolites were earlier than the parent compounds, this may indicate that the products are smaller and less hydrophobic.

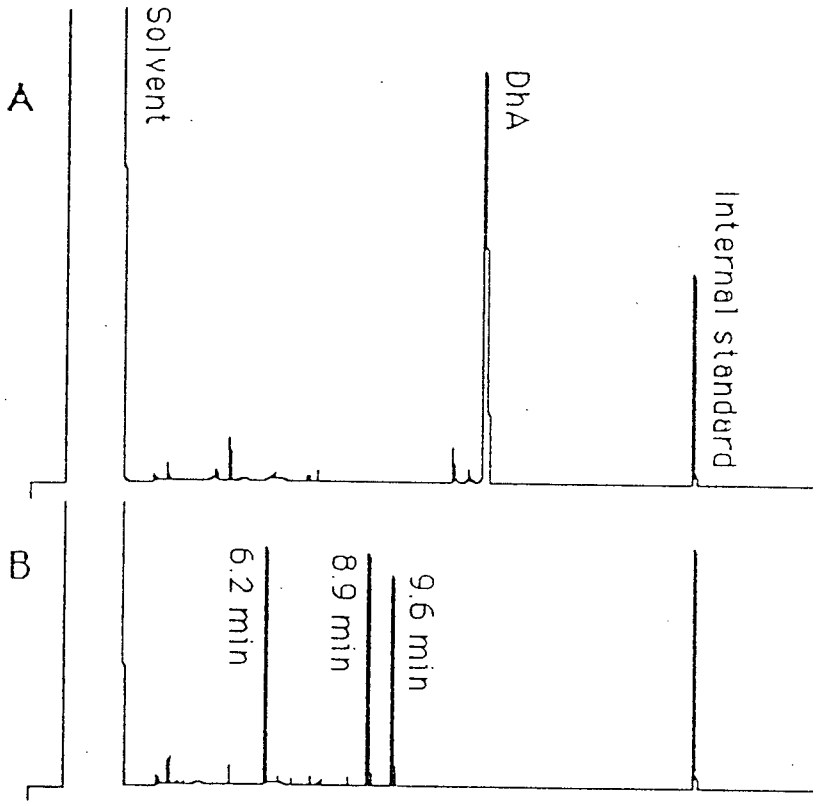


Figure 14: Products formed during the removal of DhA by a cell suspension (Table 5) of strain IpA-2; gas chromatography analyses prior to incubation (A) and after eight hours incubation (B).

DISCUSSION

This thesis reports the first microbial strains IpA-1, IpA-2 and IpA-13, isolated on IpA (Fig. 1) as a sole organic carbon and energy source (Tables 3 and 4). These results differ from previous findings in which resin acid-degrading microorganisms were isolated on only the abietanes, AbA or DhA, as the sole organic carbon source [3, 5, 10, 11, 32]. Moreover, growth on IpA was shown to be correlated to IpA removal for two of these isolates (Figs. 5A and 5B). Although this correlation was not demonstrated for IpA-13, IpA removal was shown to be associated with growth of IpA-13. These findings contrast with three other studies which demonstrated that microorganisms could transform [23] or remove [35, 52] IpA, but did not demonstrate growth on IpA.

This study could not conclusively prove that these IpA-degrading isolates completely mineralized IpA as a result of growth on the substrate. The growth curve data showed that the three IpA-degrading strains converted 24 to 34% of the IpA to protein (Fig. 5). However, the growth yield experiments showed the bacteria convert less than 25% of the IpA to protein (Fig. 9). When these protein values were converted to cell biomass, the percent conversion of IpA to cell biomass fell below the expected approximate theoretical value of 50% conversion when complete mineralization of a substrate occurs. The higher percentage conversions observed in the growth curve data may have been due to higher aeration received by the cultures when 1-ml volumes were used, compared to 12-ml for the growth yields. The comparison of IpA-2 and IpA-1 at the same temperature may also have limited IpA-2 growth yield values. When IpA-2 was grown at 20° it was shown to have higher conversion rates and converted at least 46% of IpA to cell biomass (Figs. 7A and 7B). However these latter values were suspiciously high, possible because the standard curve had a lower degree of slope. The above protein values might appear lower if calculated using the same protein standard curve as used for the other experiments.

Therefore, temperature was not necessarily a factor. Moreover, when the carbon mass balance determinations were done in the presence of excess oxygen, the data showed that two thirds of the IpA carbon was converted to biomass and CO_2 (Fig. 10), while the remaining one-third was DOC. Therefore, complete mineralization of IpA by these IpA degraders could not be proven; however, this study showed that these IpA degraders partially mineralized IpA to biomass and CO_2.

Although no conclusion can be made about complete mineralization of IpA, there are at least two possible explanations for the presence of one-third of the carbon as DOC (Fig. 10). One possibility is the IpA was completely degraded, and the DOC might contain secreted products or cellular components from lysed cells. It was hypothesized that these secreted products or cellular components might be biosurfactants, because a pause in growth and IpA disappearance occurred just before the apparent solubilization of IpA (Fig. 5). However, σ_{20} and τ_x measurements for IpA-1 did not support this hypothesis. The only surfactant activity measured was due to IpA itself (Figs. 11 and 12). These findings are consistent with a later study that grew a mixed microbial population of DhA degraders on DhA, in which no surfactant activity other than the expected surfactancy of the DhA, alone was observed [54]. Although my study showed a lack of surfactant activity for IpA-1, no general conclusion can be made about all IpA-degrading strains, as only one of the IpA-degrading strains was tested. In addition, this test was a preliminary trial without replicates. The data were also highly variable due to IpA build-up on the micropipette used to create the bubbles. This build-up continually changed the diameter of the micropipette, so an estimated diameter was used to calculate dynamic surface tension. Although this study does not support the hypothesis of biosurfactant production, more experiments need to be done before it can be conclusively disproved or validated. Production of exopolysaccharides by these

organisms might also account for the DOC component, however this avenue was not investigated. A second possibility for the presence of DOC is simply that the IpA was incompletely degraded and the DOC component contained partial degradation products of IpA. The IpA-degrading strains were shown to convert two-thirds of the IpA carbon to biomass and CO_2 (Fig.10) which would require the cleavage of a minimum of two of the three rings. If all the IpA was cleaved in this manner, the largest metabolite would be seven carbons. A previous study showed that when DhA was degraded under nutrient limiting conditions three such seven-carbon intermediates appeared (Fig. 2, 10a, 3a and 5a) [4]. However, in the present study no IpA metabolites were detected by the GC. A compound that is seven carbons or smaller might be more hydrophilic and thus not be extracted into the solvent phase, or the metabolite might no longer contain the carboxyl group required for derivitization. Neither of these types of compounds would have been detected by the GC. Alternatively, the organisms may have completely mineralized two-thirds of all the IpA molecules and the remaining one-third of the IpA molecules were converted to a metabolite also non-detectable by the GC.

If some of these smaller degradation products of IpA were converted to storage products such as polyhydroxyalkanoic acids for later use, then the values for incorporation of IpA carbon into biomass might be underestimated. Gram-negative bacteria, such as Pseudomonads, have been shown to synthesize one of the most well known polyhydroxyalkanoic acids, poly(3-hydroxybutyric acid), from acetyl-CoA or acetyl-CoA-generating substrates such as acetate, butyrate, gluconate and pyruvate [50]. The smaller degradation products of IpA would fit into the category of acetyl-CoA-generating substrates because they could be less than seven carbons and would most likely contain a carboxyl substituent group. If the IpA degraders had converted some of the IpA carbon to these storage products, then less would be incorporated as protein. Therefore, when the standard conversion factor of 55% of a cell is protein, which would be too

high, was used, a resulting underestimation of the actual cell biomass would occur. Therefore, these IpA degraders may have higher conversion of IpA to cell biomass than reported. The use of protein may have its limits in the presence of PHAs, however the existence of PHAs was not proved. Also, the biomass values seemed correct because for each organism the accounted carbon equaled or was greater than 100% of the initial carbon from the IpA.

This study showed the SBR not only contained IpA-degrading bacteria, but abietane-degrading bacteria were isolated as well (Table 3). The range of the resin acids that the abietane degraders grew on differed from that of the IpA degraders. The abietane degrader, AbA-1 was shown to use a narrower range of resin acids than the IpA degraders, because AbA-1 used only abietanes, such as AbA, DhA and Cl-DhA (Table 4). The use of only abietanes is consistent with previously described abietane degraders isolated from either the SBR [32] or another treatment system [3]. Interestingly, AbA-1 was shown to be identical to DhA-33 [32], a previously described SBR strain. In contrast, the IpA-degrading isolates were shown to use both the pimaranes and abietanes present in the SBR (Table 4). This demonstrated that the IpA-degrading strains used a broader range of resin acids than the abietane-degrading isolates described in this study and in previous studies [3, 32]. A later study confirmed these findings by showing soil isolates which grew on and degraded IpA, also used a broad range of resin acids, which included both abietanes and pimaranes [34]. Because of their use of more than one pimarane, the IpA-degrading isolates of this study are called pimarane degraders for the remainder of this discussion.

In general, within the two classes of resin acid degraders, pimarane and abietane degraders, individual strains seem to have unique specificities for individual resin acids. The pimarane degraders of the SBR were shown to use as few as three or as many as all of the five resin acids tested (Table 4). This difference was dependent on the number of abietanes the strains used. A later study also showed that pimarane-degrading soil isolates used as few as one or as

many as four of five of the same resin acids [34]. When the resin acid specificity of AbA-1 was compared to the previously described abietane degraders, the only differences were the use of palustric acid and Cl-DhA by the abietane degraders. Use of palustric acid was unfortunately not tested for AbA-1. Since AbA-1 is nearly identical to DhA-33 [32], which used palustric acid, AbA-1 might also use this resin acid. Use of Cl-DhA appears to be a more specialized characteristic of only a few strains. This includes both abietane and pimarane degraders. Two isolates from the SBR, AbA-1 (Table 4) and DhA-33 [34], were the only abietane degraders shown to both grow on the Cl-DhA mixture, and remove both 12, and 14-Cl-DhA. AbA-5 was the only pimarane degrader to do the same. All other previously described isolates, which include SBR isolate DhA-35 [34] and isolates from another treatment system [3], only removed 60 - 88% of one of the congeners, 12-Cl-DhA. The existence of both abietane and pimarane degraders that grew on and removed both 12- and 14-Cl-DhA might contradict a previous conclusion that the C-14 position must remain unchlorinated for biodegradation of DhA to occur. However, this study only showed that 12- and 14-Cl-DhA were removed as a mixture; therefore 14-Cl-DhA might only be transformed, not degraded. This may suggest that different degradation pathways exist that vary in tolerance of the placement and number of chlorine substituents.

Although the pimarane-degrading isolates of the SBR grew on both pimaranes and abietanes (Table 4), the data suggest that they are better adapted to using IpA. The strains were shown to grow on and be induced faster in the presence of IpA (Tables 4 and 5). Furthermore, my results show that IpA-1 and IpA-2, grown on DhA or PiA, produced apparent metabolites, three from DhA (Fig. 14) and one from PiA. This indicated that neither of these two substrates was completely mineralized, and therefore less potential energy would be available to these organisms growing on these substrates. In contrast, this study showed these strains not only grew on IpA, but IpA was efficiently removed because growth was shown to be correlated to IpA

removal (Fig. 5). The existence of these IpA-degrading bacteria in the SBR does not support the hypothesis that IpA was primarily removed through the fortuitous IpA-degradation by the abietane-degrading bacteria of the SBR. This study, rather, suggests that these IpA degraders are the primary bacteria responsible for IpA removal in the SBR. However, other microorganisms that could not be isolated, may also contribute to IpA removal in the SBR.

The isolated resin acid degraders of the SBR preferentially use specific classes of hydrophobic compounds, such as resin and fatty acids, over other substrates likely present in the SBR. The substrate tests showed all five strains, whether isolated from the SBR at 20°, 30° or 40°C, grew on greater than 67% of the resin and fatty acids tested (Table 4), whereas these strains were shown to grow on a maximum of 33% of the aromatic class of hydrophobic compounds. In addition, individual strains used less than 50% of the other substrates tested; included within this limited group are a few wood sugars, and likely fermentation products such as ethanol and acetate. The preferential use of hydrophobic compounds, such as resin and fatty acids, by resin acid degraders is consistent with results from a previous study which examined the substrate range of DhA-degraders from the SBR [32]. Other research on resin acid degraders [11] also showed limited use of other substrates, particularly wood sugars, such as arabinose, xylose, glucose, galactose and mannose.

Comparisons of the substrate ranges, resin acid specificities, and temperature ranges of the five resin acid-degrading SBR strains, supports the hypothesis that increasing the operational temperature of the SBR would result in a change of the resin acid-degrading bacterial community. When the SBR was operated between 20 and 30°C, all the isolates were gram-negative bacteria. The biochemical characterization of the four strains, AbA-1, AbA-5, IpA-1 and IpA-2, showed each strain belonged to one of two classes of resin acid degraders, either abietane or pimarane degraders (Table 4). These four isolates were shown to use a variety of the other substrates

besides the resin acids and fatty acids. All used fermentation products, including ethanol. Each strain used a few of the wood sugars, but used no more than one of the plant compounds (other than resin and fatty acids) tested. Lastly, the isolates maximum growth temperatures ranged between 30° and 37°C. When the SBR temperature was increased to 40°C, the majority of the strains isolated were gram-positive bacteria. When the sole gram-positive isolate, IpA-13, was tested for substrate use, it was shown to use both the pimaranes and the abietanes equally. However, only a limited range of the other likely SBR substrates, other than resin and fatty acids, were used. These included mannose, acetate, pyruvate, two plant compounds and benzoate. Also, IpA-13 had a higher maximum growth temperature of 40°C. Three gram-negative bacteria were isolated from this SBR, and subsequently lost during storage at - 20°C. However, preliminary substrate tests indicated that these strains were different from any previously isolated gram-negative strain. Therefore, the general conclusion that increasing the SBR temperature to 40°C changes the resin acid-degrading population is valid.

Interestingly, since IpA-13 occupies a specialized niche within the SBR, the organism's two-phase-growth cycle (Figs. 6) may be one mechanism for its survival in the SBR. IpA-13 may lie dormant in a resting phase until substrates become available, at which point it would grow rapidly by elongating and dividing. When the usable substrates are totally consumed, the organism would then convert back into a resting form until more nutrients become available. This observed growth cycle and possible dormancy is supported by the correlation of the 10-12 h lag shown for growth of IpA-13 on IpA (Fig. 5C) and a 10-h lag observed in resin acid removal in the SBR operated at 40°C [17].

Differences in substrate ranges, resin acid specificities and temperature ranges suggest that partially overlapping niches exist within the resin acid-degrading community of the SBR operated between 20° and 30°C. The four representative isolates, AbA-1, AbA-5, IpA-1 and IpA-2 were

either shown to use abietanes, or to preferentially use pimaranes (Table 4). Therefore, at SBR temperatures at or below 30°C, at least two resin acid-degrading isolates were found that occupy separate catabolic niches that included either use of abietanes or use of both abietanes and pimaranes. These niches are further distinguished when the resin acid specificities and substrate ranges of each organism are considered. Since each proposed niche includes the use of multiple substrates, these niches will partially overlap. For instance, besides resin and fatty acids all the strains use the same fermentation byproducts, such as acetate and ethanol. The existence of these partially overlapping niches may provide an efficient, resilient, resin acid-degrading community in the SBR.

Any conclusion about IpA-1's and IpA-2's dominance in the SBR is speculative. IpA-1 and IpA-2 might compete differently for IpA depending on the operational temperature of the SBR. This thesis showed that when the SBR was being operated at 30°C, the primary pimarane degrader most likely was IpA-1. Growth curves showed IpA-1 grew faster (Fig. 5) and protein yields were near equal or higher (Fig. 9) on the same amount of IpA, than IpA-2. When the SBR was operated below 30°C, IpA-2 may play a more dominant role in IpA removal because one of the maximum specific IpA degradation activities for IpA-2 grown at 20°C was 123% higher than the activity at 30°C (Fig. 8). However, no solid conclusion can be made about the resin acid removal dominance of IpA-2 or IpA-1 for several reasons. First, no other temperature growth curves were done for IpA-1 to compare to IpA-2 as it was discovered that no meaningful data would be gained because pauses in growth and IpA degradation prevented the calculation of growth and degradation rate constants. Second, the protein values for the IpA-2 growth curves at 20°C (Figs. 7A and 7B) were suspiciously high. These high values were most likely due to the protein standard curves being flatter than the standard curves done for the 25° and 30°C samples. Third, the maximum specific activities were highly variable because only a single measurement

was taken between two time points and used to determine the maximum specific activity. Therefore, the curves were not compared at the same timepoint. Also, high protein values for the two IpA-2 curves would result in an underestimation of the actual maximum specific activity.

As stated previously, the presence of both pimarane and abietane degraders suggests that at least two distinct resin acid-degrading bacterial populations must exist in the SBR for complete removal of resin acids at temperatures between 20° and 30°C. However, additional populations, such as PiA degraders may be needed to completely degrade PiA, because data showed that the pimarane degraders isolated did not completely mineralize PiA. Other resin acid-degrading populations may be present, as this study only tested the ability of isolates to grow on and remove five of the resin acids, which included the three primary ones in the SBR. On the other hand, these two populations may be sufficient because induction studies suggested IpA-2 cometabolically removed AbA, suggesting these resin acid-degrading bacterial populations may remove additional resin acids that they do not grow on. The general requirement of at least two distinct populations for efficient resin acid removal when the SBR was operated between 20° and 30°C means resin acid breakthrough in the treatment system might occur. Breakthrough could occur when different resin acids are released into the treatment system, such as a result of the pulp mill changing the wood species. Breakthrough would last until the required abietane-degrading, pimarane-degrading or other resin acid-degrading population is established in the treatment system.

This thesis was not only concerned with investigating the type of resin acid degraders that existed in the SBR, but also examined the resin acid removal mechanism itself. The findings from the induction studies support the hypothesis that degradation of the three primary resin acids of the SBR, IpA, DhA and AbA requires induction by at least one of these three resin acids. Two pimarane degraders of the SBR, IpA-1 and IpA-2, were shown to possess an inducible

degradative enzyme activity (Table 5 and Fig. 13) required for resin acid removal. Similarly, a previous study showed the abietane-degrading isolates from the SBR possess inducible AbA and DhA removal systems [32]. The requirement of de novo protein synthesis for DhA degradation [3] also supported the hypothesis that induction is required. In addition, this thesis shows that IpA-grown cells removed three other resin acids, PiA, DhA and AbA. This suggests one enzyme system in IpA degraders is responsible for the removal of all four of these resin acids. One possiblity is that the enzyme system has an "upper" and "lower" pathway. For instance, if the upper pathway cleaved the aromatic rings of DhA and the lower pathway cleaved the non-aromatic rings, then a compound such as IpA would be able to induce complete removal of the all the resin acids except DhA. DhA would have metabolites with aromatic rings. Interestingly, this study showed removal of DhA by pimarane degraders yielded stable metabolites (Fig. 14), while three other non-aromatic resin acids, IpA, PiA and AbA did not produce any stable, detectable metabolites. An alternative theory is that two separate pathways exist which happen to both be induced in the presence of IpA. Focusing back on the original goals of this study, this evidence and previous evidence supports a more specific hypothesis: resin acid-degrading bacteria of the SBR require the presence of at least IpA to have pimarane removal occur, whereas abietane removal can be induced in the presence of either a pimarane or an abietane. This requirement of enzyme induction for the resin acid-degrading populations provides additional valuable information for operation of a treatment system. One possible way to prevent resin acid breakthrough in the treatment system, which may occurs when the wood species is changed in the pulp mill, would be to spike the mill process water with small amounts of a particular resin acid, before that resin acid is actually released into the process water. This would preinduce the required enzymes within the resin acid-degrading population and thus the bacteria would have all the enzyme machinery in place to remove the desired resin acid.

Looking at the bigger picture of the operation of the SBR at temperatures ranging from 30°C to 50°C, this thesis also assessed how temperature affected the overall community of resin acid-degrading organisms in terms of their numbers and their resin acid specificity. The MPN data showed similar populations of IpA-degrading organisms and a ten-fold increase in the populations of DhA and AbA-degrading organisms when the SBR was increased from 30° to 40°C (Fig. 4). When the operational temperature of the SBR was increased above 40°C, the number of resin acid-degrading organisms decreased from 10^7 degraders per 100 ml to only 10^3 degraders per 100 ml, for each of the three resin acids. This decrease is supported by the failure to isolate any resin acid-degrading strains from the SBR at 45°C and 50°C (Table 3). This failure occurred even though two different enrichment or isolation methods were used.

The above objective tied directly into one of the objectives of a previous thesis [17], which was to determine how temperature affected the removability of resin acids from the SBR. Therefore it was of interest to see if the data from this study corroborated any of the previous study's conclusions and provided additional insight into some of the findings. Efficient removal of resin acids from the high strength influent in the SBR at 30°C and 40°C was supported by my MPN findings, which showed that a minimum of 10^7 AbA-degrading, DhA-degrading and IpA-degrading organisms per 100 ml existed in the SBR at both temperatures (Fig. 4). These findings may be on the conservative side because an earlier study showed a ten-fold higher number of DhA-degrading organisms existed in the 30°C SBR [32]. Also, the MPN method does not account for the non-culturable organisms in the SBR because the method only enumerated culturable organisms. Therefore, an even larger number of resin acid degraders may exist. When the SBR operating temperature was increased to 45° and 50°C, decreases in the SBR biomass inventories occurred. The MPN results supported these findings. An overall reduction in the number of IpA, DhA and AbA degraders in the SBR at 45°C and 50°C was shown. This

decrease provides one answer to the question of why poor removal of resin acids occurred in the SBR at both 45°C and 50°C. The MPN findings also suggest that resin acid removal at the higher temperature SBR required the presence of another substrate. A population of 10^5 IpA degraders and DhA-degraders per 100 ml was shown to exist which removed both resin acids in the presence of the evaporator concentrate, while no population existed that could use resin acids as sole carbon sources. Lastly, the conclusion that higher temperatures made maintenance of the active biomass difficult was supported by an inability to isolate organisms at the higher temperatures (Table 3). Also, MPN findings showing the number of culturable resin acid-degrading organisms in the SBR at 45°C and 50°C SBR decreased from 10^7 to 10^3 degrader per 100 ml supported this conclusion. These MPN values did differ from the smaller half-log reduction observed for the volatile suspended solids measurement of the SBR biomass [17]. However, this study's values may more accurately reflect the living biomass population because the VSS measures both dead and live cells.

Up until this point, this discussion has focused on specific resin acid-degrading organisms within the SBR, and how changes in the operational temperature affected these organisms and the resin acid-degrading community as a whole. This last section will address how preliminary evidence from this study supports the hypothesis that the SBR biomass is resilient to rapid changes in either pH or resin acid concentration. However, this resilience was shown to be dependent on the degree, rather than the type of flux the treatment system experienced. No inhibitory effect on the resin acid removal ability of the SBR biomass was observed when the biomass experienced a thirty-fold increase in resin acid concentration up to 3000 μM (0.9 g/l) (Table 1). Also, a one unit increase or decrease in pH, to 6 or 8 (Table 2) had no inhibitory effect. However, a two unit increase in pH inhibited the biomass resin acid removal ability by over 50 percent. Although an inhibitory effect was observed at pH values of 5 and 9, no

conclusion can be made whether the inhibition was due to a direct pH effect, or an indirect effect such as resin acid toxicity caused by a higher solubility of resin acid at pH 9. These pH effects are consistent with a previous study which demonstrated that pH values between 6 and 8 had no inhibitory effect on resin and fatty acid removal in a laboratory scale treatment system [29]. The conclusion is further supported by a study which showed rapid pH shifts greater than one unit disrupted the biochemical efficiency of substrate removal [13]. In addition, a resilience to high concentrations of resin acids has been shown for individual isolates of different treatment systems. Growth of IpA-1 and IpA-2 of this study was not inhibited by 600 μM (0.18 g/l) of IpA (Figs. 9A and 9B). Similarly, five DhA degraders isolated from bleach kraft mill effluent tolerated concentrations up to 600 μM DhA [3]. However, the degree of resilience to high resin acid concentrations or fluxes in pH may also depend on the organisms or on the treatment system itself. One study showed resin acid concentrations as low as 133 μM inhibited the growth of 54% of the bacterial strains tested [14]. The same study showed activated sludge only removed resin acids when the pH was above 7.3. These differences between organisms may suggest that resin acid degraders are more tolerant of higher resin acid concentrations compared to non-resin acid degraders because these previous cited strains were not proven grow on resin acids a sole carbon source, although they were isolated from wood sources.

Interestingly, some of the data shown here appears to be at variance with that reported by Lo *et al.* [29]. They showed efficient resin and fatty acid removal down to a pH of 5. The most plausible explanation for this variance is that the treatment system [29] was acclimatized to each pH and the treatment system had a 3-day hydraulic rentention time. This was much different from the biomass experiment (this study) in which the pH was rapidly changed, and resin acid removal was limited to a twelve hour period. The performance discrepancy between an acclimatized system and one that has experienced a rapid flux in an operational parameter has also

been reported by Johnson [17]. When the SBR was acclimatized to temperatures of either 30^0 or 40°C, it efficiently removed resin acids. When the temperature was increased from 37 to 39°C in one day, the resin acid removal decreased by half. This observed variance between the biomass resting cell data (this study) and the acclimatized pH study [29] indicates the resting cell assay provides a reliable and controlled method for measuring how rapid changes in the operation of the SBR will effect resin acid removal. Although the resting cell assay is a good method, the data would have been more accurate and reproducible if the assays were done in individual tubes, which were then wholly extracted at each time point. This latter method was used for the growth curves and the cell suspension assays with pure cultures, and was shown to have less variability among triplicate data values compared to that of the subsampling method.

CONCLUSIONS

Five resin acid-degrading bacterial strains were isolated from the SBR at either 20° or 40° and were tested for substrate use, resin acid use and temperature range. IpA-1, IpA-2, IpA-13, and AbA-5 are the first reported isolates which grew on IpA as a sole organic carbon and energy source. Growth yields and carbon mass balance determinations for IpA-1, IpA-2 and IpA-13 did not conclusively prove that IpA was completely mineralized because one-third of the IpA carbon remained as DOC, however partial mineralization of IpA was shown. No conclusion could be made about whether the DOC component was a biosurfactant produced by IpA degraders; however, preliminary data showed IpA-1 did not produce a biosurfactant. The presence of IpA degraders that are better adapted to using IpA than other resin acids, suggests that IpA was degraded in the SBR by these IpA degraders, not fortuitously degraded by the abietane degraders. These IpA degraders having an induced degradative enzyme system, which removes not only IpA but also PiA, DhA, and AbA, also supports the hypothesis that in order for the IpA degraders to remove the three primary resin acids in the SBR, IpA would be required to be present for induction of the organism's enzyme system.

Biochemical characterization of the five strains showed that increasing the SBR from 20° to 40°C changed the culturable resin acid-degrading bacterial population. At 20°C at least two resin acid-degrading populations, a pimarane-using one and an abietane-using one, existed which may also have used some of the other available SBR substrates. When the temperature was increased to 40°C, the population included a gram-positive bacterium, IpA-1, which used pimaranes and abietanes equally, but used fewer of the other substrates likely present in the SBR. Increasing the SBR temperature above 40°C decreased the population of culturable AbA, DhA, and IpA-degrading microorganisms from 10^7 per 100 ml to 10^3 per 100 ml. The remaining populations required an additional energy source to remove resin acids. In addition, the resin acid-degrading biomass of the SBR was resilient to rapid changes in pH between 6 and 8, and resin acid concentrations up to 3000 μM (0.9 g/l).

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