Communications to the Editor

Microbially Produced Rhamnolipid as a Source of Rhamnose

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The sugar L-rhamnose (6-deoxy-L-mannose) is used as a fine chemical in scientific and industrial settings, as a component in chemical reactions, and as a starting material in the synthesis of organic compounds. Present methods for the commercial preparation of rhamnose require extraction of quercitrin from oak bark, naringin from citrus peels, or rutin (quercetin-3-rutinoside) from oak bark or a variety of plants, such as buckwheat.[1,2] Quercitrin is a glycoside made up of the aromatic aglycone quercetine and rhamnose, whereas naringin and rutin are glycosides containing a glucose moiety in addition to rhamnose and the aglycone. Rhamnose is recovered by the hydrolysis of these molecules. Several disadvantages accompany the labor-intensive processes for production of rhamnose from quercitrin, naringin, and rutin, including the production of large quantities of potentially toxic, aromatic waste products and the need for toxic or corrosive chemicals in the extraction process. Furthermore, the bulky raw materials used for extraction must be harvested and then either transported at some expense to the extraction facility, or the processing plant must be located in proximity to the raw materials.

Another potential source of rhamnose is rhamnose-containing polysaccharides produced by plants or microorganisms. Certain polysaccharides (i.e., gelrite) are characterized by gelling or thickening properties,[3] while others may be used as emulsifiers.[4,5] The growth of certain microorganisms results in the production of extracellular polysaccharides.[6] These extracellular polysaccharides may be present as a capsule around the outside of the cell or may be released into the growth medium as slime, or may be present in both capsular and released form. For instance, production of a *Pseudomonas elodea* polysaccharide containing 30% rhamnose was reported at yields of 1.5% "gel" from growth in 3% glucose.[7] Fermentation of *Acinetobacter calcoaceticus* has also been reported to yield 0.5 g/L polysaccharide containing 80% rhamnose.[8] Yields as much as 3.5 g/L rhamnose have recently been reported

from polysaccharides obtained from specific strains of *Acinetobacter* and *Klebsiella*.[6]

One major disadvantage to the production of rhamnose from polysaccharides is that isolated polysaccharides contain other sugars in addition to rhamnose. Although acid hydrolysis of such polysaccharides yields rhamnose, the rhamnose product is often contaminated with the other sugars that are present in the polysaccharide.

A second disadvantage arises from an inherent property of polysaccharide itself. If microbial strains selected for elevated production of exopolysaccharide are employed as the source of rhamnose-containing polysaccharide, the release of polysaccharide into the growth medium results in a culture with high viscosity. This viscous suspension requires additional amounts of energy for the necessary agitation of the culture medium. In addition, the delivery of desired amounts of oxygen or of additional nutrients becomes increasingly difficult in a viscous medium. The high viscosity of the culture also makes the isolation of the polysaccharide very difficult.

One final disadvantage is that the polysaccharides released into the culture medium by microbial cells often copurify with protein components of the culture medium or with proteins produced by the cultured cells. These protein contaminants require the use of additional purification steps prior to hydrolysis of the polysaccharide. Upon isolation of polysaccharides from plants or microbes, the processing of polysaccharides requires dissolution in solvents. This polysaccharide–solvent mixture is also a highly viscous material that is difficult to manipulate. Thus, all the problems associated with the processing of rhamnose from polysaccharides may result in making this approach unsuitable for large-scale production.

The preparation of rhamnolipids, effective as surfactants and emulsifiers, has been previously described.[9,10] Efficient production of rhamnolipids from microbial sources generally employs a culture medium containing excess levels of carbon and phosphorus, but limiting concentrations

of nitrogen and/or trace metals. A water-soluble carbohydrate, such as glucose, is usually used as a carbon source and the optimal rhamnolipid production is obtained from resting cells. Typical yields of rhamnolipids according to these conditions range 0.5–2.25 g/L using continuous culture conditions[11-13] and 15 g/L using resting cells.[10]

This communication describes a method for the production of high levels of rhamnolipid from *Pseudomonas aeruginosa* in a defined culture medium containing corn oil. The isolated rhamnolipid is then hydrolyzed to produce a mixture of rhamnose and a fatty acid, 3-hydroxydecanoic acid, which are easily separated.

MATERIALS AND METHODS

Microorganisms

The organism (strain UI29791) was isolated from a patient at the University of Iowa Hospitals and Clinics and tentatively identified as a *Pseudomonas* strain by: a positive oxidase test with tetramethyl-*p*-phenylenediamine;[14] its morphological appearance as Gram negative rods;[15] and a battery of nutritional tests (Vitek Analysis System, McDonnell–Douglas Corp.). Upon positive identification of the isolate as *Pseudomonas* the strain was routinely stored on nutrient agar slants at 37°C and transferred once a month. This *Pseudomonas* strain was found to produce high levels of rhamnolipid in shake flask experiments and was further characterized using the API Analysis System (Analytab Products, Plainview, NY). It was determined to be a member of the fluorescent group and its ability to grow at 42°C permitted it to be speciated as *Pseudomonas aeurginosa.*[14]

Culture Growth in Shake Flasks

Pseudomonas aeruginosa was grown in 500-mL shake flasks containing 50 mL defined media consisting of the following salts (in g/L): sodium nitrate, 15; potassium chloride, 1.1; sodium chloride, 1.1; iron (II) sulfate · 7H₂O, 0.00028; calcium nitrate · 4H₂O, 0.01; potassium dihydrogenphosphate, 3.4; potassium hydrogen phosphate, 4.4; and magnesium sulfate · 7H₂O, 0.5. To 1.0 L of this mixture 5 mL of trace salt solution was added containing (in g/L): zinc sulfate · 7H₂O, 0.29; calcium chloride · 4H₂O, 0.24; copper (II) chloride · 6H₂O, 0.24; copper (II) sulfate · 5H₂O, 0.25; and manganese (II) sulfate · H₂O, 0.17. The salt solution was adjusted to pH 6.5 and 75 g/L corn oil was added. Modifications in this basic media were made to study the nutritional requirements of these organisms as described in the text. The shake flasks were inoculated and incubated at 37°C on a gyratory shaker at a rate of 200 rpm. After six days the culture reached the desired cell density. To determine dry cell weight samples were removed and the cells were recovered by centrifugation and the cell pellets obtained were resuspended in water, transferred to preweighed containers, and dried in an oven at

100°C to constant weight. The culture was centrifuged at 6000*g* for 30 min at 4°C, the supernatant was collected and rhamnolipid concentration was determined by measuring rhamnose concentration in the culture supernatant.

Culture Growth in Fermentors

Pseudomonas aeruginosa was grown in a 14-L fermentor containing 6 L defined medium having the same composition as used in the shake flask experiments. Modification of this media or the addition of components during the fermentation are described in the text. The culture was incubated at 37°C, and the pH was maintained between 5.5 and 7.0 by the addition of either 1*M* sodium hydroxide or 1*M* sulfuric acid. An aeration rate of 0.5 vvm, a dissolved oxygen level of 25–90% saturation, and an agitation rate of 450–550 rpm were maintained. Antifoam B (Sigma Chemical Co., St. Louis, MO) was added during the fermentation using a foam controller system to a maximal final concentration of 10 mL/L. Samples were periodically taken over 10 days of culture. At the end of the fermentation the culture suspension was centrifuged (45 min at 6000*g*) to remove cells, and the supernatant was adjusted to pH 2.5 with sulfuric acid prior to storage overnight at 4°C. This solution with its resulting precipitate was centrifuged as above in order to separate a precipitate of rhamnolipid.

Using the same medium and culture volume, *Pseudomonas aeruginosa* was grown by semicontinuous fermentation. Over a four-day incubation period, 1.5 L (one-fourth the working volume) of culture medium was removed and replaced with fresh medium at 47 and 75 h and the rhamnolipid was recovered from the total 6 L of culture supernatant as previously described.

Recovery and Hydrolysis of Rhamnolipid

The rhamnolipid precipitate obtained from the culture media cell-free supernatant was resuspended in 1*M* sulfuric acid (at a concentration of 1 g rhamnolipid/100 mL sulfuric acid) and heated for 2 h at 100°C. This hydrolysis mixture was extracted with four volumes of ethyl acetate, yielding free rhamnose in the aqueous layer and any residual rhamnolipid along with 3-hydroxydecanoic acid in the organic layer.

Alternatively, the cell-free supernatant from the culture media acidified to pH 3, or the precipitated pellet suspended in water at pH 3, was extracted with four volumes of ethyl acetate. After the drying of over anhydrous magnesium or sodium sulfate the ethyl acetate was removed under vacuum to obtain rhamnolipid as a clear yellow oil which could then be hydrolyzed as described previously to obtain rhamnose.

Analysis of Rhamnolipid and Rhamnose

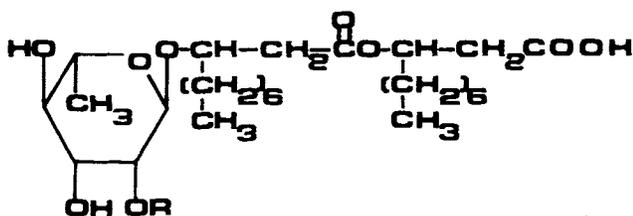The rhamnolipid extracted by the above procedure represents a mixture of rhamnolipid containing either one or

Figure 1. Structure of Rhamnolipids isolated from *Pseudomonas aeruginosa*, where R = H and R = rhamnosyl.[9,10]

two rhamnose residues (Fig. 1), which was separated by column chromatography on silica gel eluting with methylene chloride/methanol (80/20). Such a mixture was previously obtained from another *Pseudomonas aeruginosa* strain.[9,10] Both rhamnolipids were characterized by fast atom bombardment mass spectrometry as well as proton and carbon nuclear magnetic resonance spectroscopy (NMR) in deuteromethanol. The rhamnose content of the pure rhamnolipid obtained by organic solvent extraction or the rhamnolipid dissolved in culture medium was determined following acid hydrolysis by colorimetric assay.[16]

RESULTS AND DISCUSSION

Pseudomonas aeruginosa isolate UI29791 produced an emulsifier when grown in liquid culture. This emulsifier could be recovered by the ethyl acetate extraction of the cell-free spent medium.

The growth of *Pseudomonas aeruginosa* strain UI29791 and its production of rhamnolipid was examined in shake flasks using medium containing glucose, corn oil or glucose and corn oil as carbon sources. The results in Table I indicate that corn oil serving as the sole carbon source produces significantly increased levels of both cellular mass and rhamnolipid. The increase in rhamnose cannot be attributed solely to the presence of more cells, since cell mass was 2.3 times greater when corn oil alone was used as the carbon source, while rhamnose levels were ca. 11–27 times greater when corn oil was used. In addition to corn oil, other vegetable oils including soybean oil, coconut oil, and cottonseed oil result in similar cell growth and rhamnolipid production. Corn oil is readily available at low cost and its use as a carbon source in the absence of glucose avoids potential sugar contamination of the rhamnose product.

Table I. Growth of *Pseudomonas aeruginosa* on various carbon sources.

Carbon source[a]	Rhamnose (g/L)	Dry cell weight (g/L)
75 g/L glucose	0.5	2.3
40 g/L corn oil	5.4	5.2
37 g/L glucose + 20 g/L corn oil	0.2	2.3

[a] Medium is identical to the shake flask media described in the experimental section, except for the following modifications: NaNO₃ (12.2 g/L); KH₂PO₄ (6.8 g/L); K₂HPO₄ (8.7 g/L); pH 7.5. The only carbon source present in the growth medium is that indicated in Table I. The values were obtained with a 140-mL culture.

Both reduced and oxidized forms of nitrogen could support the growth of this organism. The effect of limiting concentrations of nitrogen (as nitrate) on the production of rhamnolipid was examined. Nitrogen limitation had been reported to increase the yield of rhamnolipid produced by *Pseudomonas aeruginosa*.[11] However, in preliminary experiments with our strain, we found that medium containing low nitrogen levels (4.2 g/L sodium nitrate, representing 0.7 g/L nitrogen) produced less cells and rhamnose than medium containing over 2 g/L nitrogen. The results of further shake flask experiments show that low levels (1.5–2 g/L) of nitrogen result in enhanced production of rhamnolipid early in the fermentation but the addition to concentrations of 2–3 g/L nitrogen late in the fermentation results in higher cell growth and thus higher overall yields of rhamnolipid (Table II).

The concentration of iron has been suggested to play a role in the regulation of rhamnolipid production by *Pseudomonas aeruginosa*.[11] The results of shake flask experiments carried out to test the effect of iron concentration on rhamnolipid production is summarized in Table II. An iron concentration of 50–100 μg/L was demonstrated to be optimal for the production of rhamnolipid. Slightly lower or higher iron concentrations resulted in a two-fold decrease in rhamnolipid production.

Experiments were also conducted to determine the optimum concentration of sulfur, magnesium, phosphorous, calcium, and trace metals. The results of these studies (Table II) showed that these elements had little effect on rhamnolipid production provided the concentrations of these essential nutrients were sufficient to support cell growth.

Once the nutritional requirements had been defined for the optimal production of rhamnolipid by *Pseudomonas*

Table II. Elements used in the experiments.

| Element[a]	Element concentration tested		Approximate element concentration for maximal Rhamnose level (g/L)
	Minimum (g/L)	Maximum (g/L)	
N	1	3.6	1.75[b], 2.5[c]
Fe	0.000025	0.0016	0.00075
S	0.014	0.12	0.08
Mg	0.004	0.21	0.07
P	0.25	1.8	1.25
Ca	0.0002	0.006	0.0002
Trace elements	0.000025	0.0009	0.0006

[a] All experiments were at 37°C in 500-mL flasks shaken as rapidly as practical, but at rates varying slightly in speed and length of stroke (due to necessary use of several different shakers; however, all flasks in a given experiment were on the same shaker). Flasks contained 50 mL medium and 10 mL inoculum grown from 36 to 60 h. Table II reflects some element carryover from the inoculum (17 vol %). For example, the inoculum medium for Mg, S, Fe, trace elements, and Ca had one-half the normal medium strength of these elements. Nitrogen was as NaNO₃; iron as FeSO₄ · 7H₂O; sulfur as K₂SO₄; magnesium as MgCl₂; phosphorous as KH₂PO₄ and K₂HPO₄; calcium as CaCl₂, and trace metals as ZnSO₄ · 7H₂O, CoCl₂ · 6H₂O, CuSO₄ · 5H₂O and MnSO₄ · H₂O (combined concentration).

[b] Initial concentration.

[c] Final concentration.

aeruginosa in shake flasks, studies in fermentors offering pH, dissolved, oxygen, and foam control were initiated in an effort to achieve high levels of rhamnolipid production. Batch fermentation of *P. aeruginosa* (Fig. 2) lasted 10 days with maximal cell density of 15 g/L observed on day 5 and a maximal rhamnolipid level achieved on day 8 of 46 g/L corresponding to a rhamnose concentration of 23 g/L. The volumetric productivity over the first eight days of the fermentation was 5.8 g rhamnolipid/L day. In an effort to improve the volumetric productivity, a semicontinuous batch fermentation was performed in which one-fourth of the working volume of the culture medium was removed and replaced with fresh medium at 47 and 75 h. The overall volumetric productivity was 6.4 g rhamnolipid/L day, similar to that in the batch fermentation. The volumetric productivity from the time of the first medium removal to the attainment of maximum cell density was 10 g rhamnolipid/L day, a rate similar to that observed in Figure 2. These results compare favorably with a recently reported productivity of 3.5 g rhamnolipid/L day for another strain of *P. aeruginosa* grown on glucose in continuous culture.[13]

The 1:1 mixture of rhamnolipids obtained (Fig. 1) is ca. 40 wt % rhamnose which compares well with the rhamnose content of extracellular microbial polysaccharides.[6] Fermentation of *P. aeruginosa* yields 25 g rhamnose in the form of rhamnolipid or 1.7 g rhamnose/g dry cell weight. The productivities of this fermentation were 25 g rhamnose/L and 15 mg rhamnose/g dry cell weight h. This compares favorably with yields of 3.5–4.0 g rhamnose/L and productivities of 2.5–4.5 mg rhamnose/g dry cell weight h reported for rhamnose production in the form of exopolysaccharides from *Klebsiella* and *Acinetobacter*.[6] In addition to vastly increased yields and productivity,
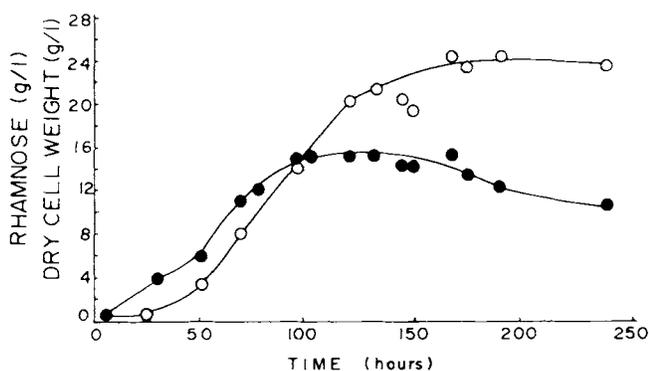
rhamnose preparation from rhamnolipid eliminates the problems associated with the polysaccharide source such as high culture viscosity, recovery and purification problems. Finally, a secondary product, 3-hydroxydecanoic acid, is obtained which may have a potential market value.

CONCLUSIONS

This communication describes the isolation and characterization of a strain of *Pseudomonas aeruginosa* which produces high levels of rhamnolipid. The nutritional requirements for rhamnolipid production were investigated and a carbon source consisting only of corn oil was found to give the best results. Batch and semi-continuous batch fermentation demonstrated that high yields and productivities of rhamnolipid could be obtained making this approach a viable method for the commercial production of rhamnose.

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Figure 2. Batch fermentation of *Pseudomonas aeruginosa*. Time in hours is plotted versus (●) dry cell weight and (○) rhamnose concentration (in g/L). At 27 and 76 h, additional magnesium iron, calcium, and trace metals were added equal to those originally present in the medium.