FEATURE ARTICLE

RHAMNOSE FROM THE PRODUCTS OF MICROBIAL FERMENTATION

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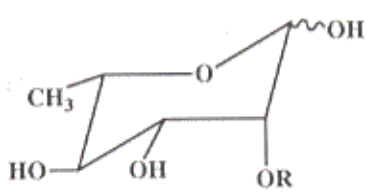
Introduction

Although they are often used as a carbon source in microbial fermentations, sugars are rarely thought of as desired end-products. Sugars are typically prepared from plant materials through extraction. An increased demand for unusual sugars requires the feasability of their microbial preparation to be re-examined.

Although there have been many major advances in carbohydrate chemistry since Emil Fisher first determined the structure of glucose in 1881, the past decade has brought some truly remarkable developments. These developments, particularly the use of sugars as chiral synthons in organic synthesis, have increased the demand for sugars.

This increased interest in carbohydrates suggests another look to be taken at methods for the preparation of unusual monosaccharides having commercial value. Fermentation offers the opportunity to prepare bulk quantities of a wide variety of chemicals at very competitive costs. This paper examines a fermentative approach towards preparing one such monosaccharide, rhamnose.

L-rhamnose (6-deoxy-L-mannose) (I) is a fine chemical used as a chemical intermediate in the synthesis of a variety of fine chemicals. Presently rhamnose is prepared by extraction of quercitin 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). Quercitin is a glycoside composed of the aromatic aglycone quercitin and rhamnose, while naringin and rutin are glycosides containing both glucose and rhamnose. Rhamnose is recovered by the hydrolysis of these glycosides. Extraction is a labor-intensive process and poses several disadvantages for rhamnose production. Extraction of plant tissues results in the production of large quantities of potentially toxic, aromatic waste products. The bulky raw materials must be harvested and transported to the extraction facility. Alternatively, processing plants must be located in proximity to the raw materials.



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One potential process to prepare rhamnose, which addresses many of these problems, would involve its production by microbial fermentation. This paper discusses two microbiological approaches for the preparation of rhamnose.

Microbial Polysaccharides As A Source of Rhamnose

Bacterial growth is often accompanied by the production of polysaccharides that are found outside the cell wall. These exopolysaccharides may be released to the environment as slime or they may be as a capsule attached to the bacteria(3). A polysaccharide may be important to the bacterium for adhesion, infection, or protection. But it may have commercial value as well. Some polysaccharides have gelling properties (i.e., agar and gelrite)(4,5), others have emulsifying properties(6,7), and still others may represent a source of certain important monosaccharides, such as rhamnose. The commercial value of a polysaccharide as a source of monosaccharides depends on its characteristic saccharide composition, the quantity produced by a culture, and the ease of harvest and processing of the polysaccharide.

Genetics are believed to determine both the saccharide composition and quantity of microbial exopolysaccharides. Culture conditions(8), however, may also influence polysaccharide production. Excess nutrient carbohydrate and low temperatures generally favor exopolysaccharide formation(3,9,10). Exopolysaccharide yield and its saccharide composition may also be influenced by limiting nutrients such as nitrogen, carbon, phosphorus, and sulfur(8). Until recently, the composition of exopolysaccharide was believed to be independent of carbon source(11,12).

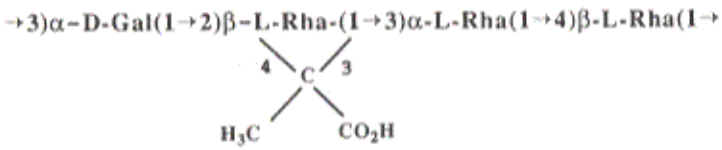
Our laboratory examined two microorganisms in an effort to prepare a polysaccharide (II) having a high

Table 1. Rhamnose content of exopolysaccharides (EPS) produced by *Klebsiella sp.* and *Acinetobacter calcoaceticus*.

	Klebsiella sp.		*A. calcoaceticus*	
Carbon Source[a]	Per cent rhamnose	EPS, g/L	Per cent rhamnose	EPS, g/L
Glucose	65	0.38	41	0.01
Mannose	66	0.28	48	0.01
Rhamnose	64	0.28	48	0.01
Succinate	47	0.08	57	0.17
Glutamate	28	0.07	62	0.26
Ethanol	33	0.02	62	0.23
Complex medium and glucose	57	1.11	22	0.61

a Fermentations were performed in shake flasks as described in reference 13.

rhamnose content(13). *Klebsiella sp.* strain K32 and *Acinetobacter calcoaceticus* BD4 both produce exopolysaccharides with a high rhamnose content(6,14). *Klebsiella sp.* strain K32 was the gift of G.G.S. Dutton of the University of British Columbia while *A. calcoaceticus* BD4 was obtained from the American Type Culture



II

Collection (ATCC 33304). The cultures, maintained on nutrient agar were grown, in shake flasks or small fermentors, on complex media(13). This media was supplemented with various carbon sources to ascertain the effect of nutrients and environmental factors on the rhamnose composition of the exopolysaccharides.

Polysaccharide composition has generally been thought to be independent of the carbon source utilized for growth(11,12), although only sugars were tested as substrates. The results presented in Table 1 show that both yields and rhamnose content of exopolysaccharides produced by both of the microorganisms examined depended on the carbon source utilized. Different sugar substrates, however, caused little variation in exosaccharide composition during growth and no variation in exopolysaccharide composition when the culture was harvested in the stationary phase. *Klebsiella sp.* grew well on all of the carbon sources but gave the highest yield of exopolysaccharides and cell mass when grown on sugars. *Klebsiella sp.* could even utilize its own exopolysaccharide as a substrate. Although *A. calcoaceticus* produced a polymer containing rhamnose, glucose, and mannose, it could not grow well on these sugars. *A. calcoaceticus* was also unable to grow when given its own exopolysaccharide as a substrate. The exopolysaccharide produced by *A. calcoaceticus* is a bioemulsifier(4,10), and its only function in nature may be to make other carbon sources (i.e., hydrocarbons) more available for growth.

Both *Klebsiella sp.* and *A. calcoaceticus* grew exponentially in fermentors producing exopolysaccharide at a nearly constant rate. Interestingly, no variation in the rhamnose content of the exopolysaccharide was seen over the growth period of *A. calcoaceticus*. The rhamnose content of *Klebsiella sp.* exopolysaccharide, however, increased from 12% in early exponential growth to 55% in the final sample (Fig. 1). A similar variation in the glucose content of an exopolysaccharide from *Serratia marcescens* during its growth has been reported(11).

The variation in rhamnose content observed during growth requires that an organism be able to produce more than one type of polymer or to modify its polysaccharide extracellularly. There is no evidence that the exopolysaccharide from *Klebsiella sp.*(15), is branched. Thus it is probably not modified after release. Rather the variation in its composition is probably the result of the production of a spectrum of different polymers. *S. marcescens*(11) and Rhodopseudomonas capsulata(16), for example, reportedly produce a spectrum of polymers.

Variation in rhamnose content can be explained by two possible mechanisms. The first is the production of similar polymers, each with a defined (but different) primary structure. The distribution of individual polymers within this spectrum might vary depending on the growth stage or carbon source. Polymer composition is measured on the gross mixture of exopolysaccharide, and thus the observed variation could be explained on this basis. The second possibility is that the primary structure of the polymer may change. For example, a rhamnose-rich polymer might result when the level of activated rhamnose is elevated within the cell.

These studies suggested that it was possible to alter the

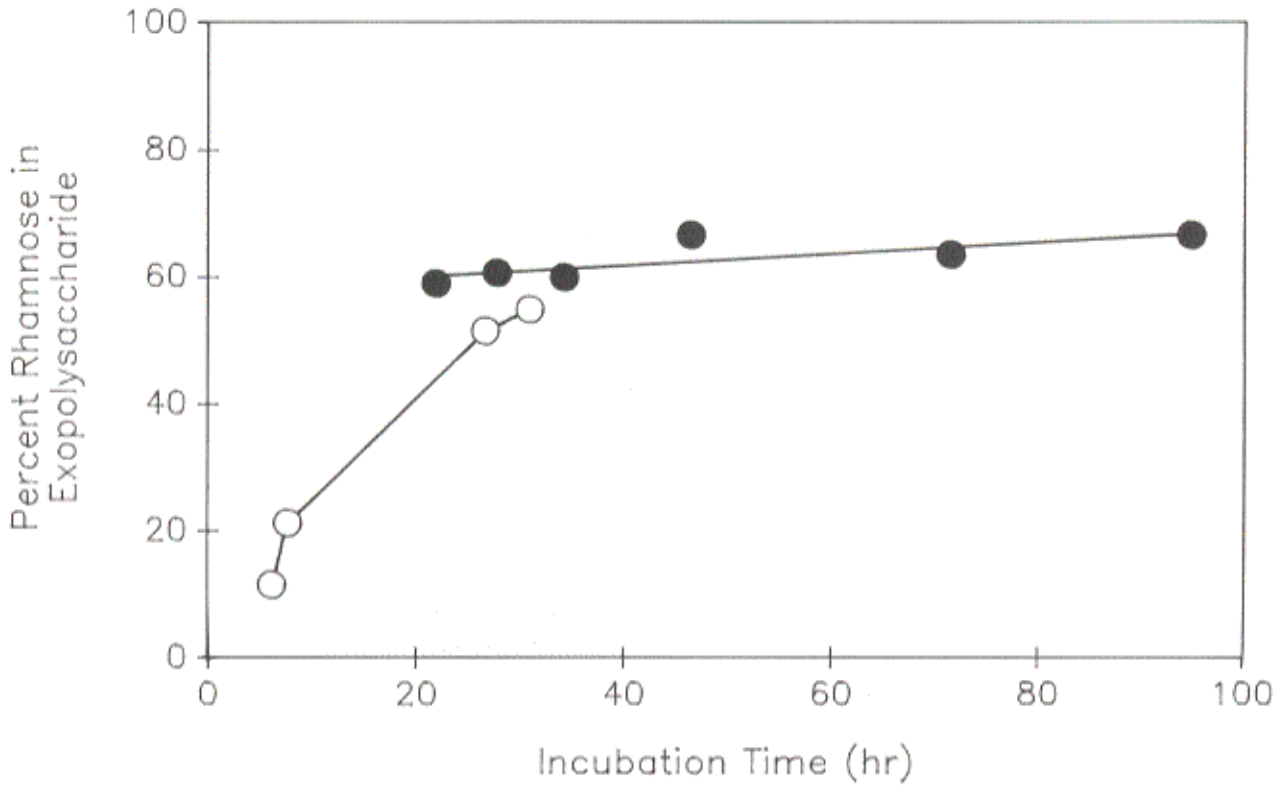


Fig. 1 Relative rhamnose composition of the exopolysaccharide produced by *A. Calcoaceticus* in a fermentor on defined medium containing succinate at 26° at pH 7.0 and by *Klebsiella sp.* in a fermentor on a complex medium containing glucose at 37°C at pH 7.0. Defined medium containing succinate gave similar results. Symbols: ○ percentage of exopolysaccharide produce by *Klebsiella sp.* determined to be rhamnose and ● percentage of exopolysaccharide produce by *A. calcoaceticus* determined to be rhamnose.

rhamnose content of an exopolysaccharide by selecting appropriate growth conditions (ie, carbon source and harvest time). The best results obtained were with *Klebsiella sp.* grown on succinate in a fermentor. This resulted in an exopolysaccharide production rate of 8 mg h^{-1} per g of dry cell weight with a total production of 6.4 g $liter^{-1}$ of exopolysaccharide at harvest.

Disadvantages with the Production of Rhamnose from Exopolysaccharides

There are certain problems associated with the production of rhamnose from exopolysaccharides. First, exopolysaccharides from organisms such as *Klebsiella sp.* or *A. calcoaceticus* contain other sugars in addition to rhamnose. Thus although acid hydrolysis of such polysaccharides yields rhamnose, it is often contaminated with the other sugars that were present in the polysaccharide.

A second disadvantage arises from an inherent property of the polysaccharide itself. Microbial strains that produce elevated levels of rhamnose-containing polysaccharide, release this polysaccharide into the growth medium resulting in a culture with high viscosity. This viscous suspension requires additional energy for the agitation to deliver adequate amounts of oxygen. The high viscosity of the culture also makes the recovery and isolation of the polysaccharide very difficult.

A final disadvantage of preparing rhamnose from exopolysaccharides 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. This necessitates additional purification steps prior to polysaccharide hydrolysis. This processing of polysaccharide requires dissolution in solvents giving mixtures that are highly viscous and difficult to manipulate. All the problems associated with the processing of rhamnose from exopolysaccharides may result in making this approach unsuitable for large-scale production of rhamnose and suggested the need to develop alternative approaches.

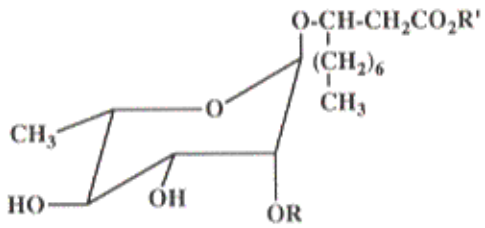
Table 2. Growth of *Pseudomonas aeruginosa* on corn oil and glucose as carbon sources.

Carbon source[a]	Rhamnose g/L	Rhamnose/Dry cell weight g/g
Glucose	0.5	0.22
Corn oil	5.4	1.04
Glucose and corn oil	0.2	0.09

a The only carbon source present in the growth medium was glucose at 75 g/L, corn oil at 40 g/L or glucose and corn oil at 37 and 20 g/L, respectively. The medium composition is described in reference 21.

Microbial Glycolipids as a Source of Rhamnose

A second potential microbial source of rhamnose are rhamnolipids. Rhamnolipids (III) have been prepared as surfactants and emulsifiers(17,18). Their efficient production from microbial sources can be influenced by manipulating environmental conditions and reportedly



III

R=H	R'=H
R=H	R'=α-L-rhamnosyl
R=CH[$(CH_2)_6CH_3$]CH_2CO_2H	R'=H
R=CH[$(CH_2)_6CH_3$]CH_2CO_2H	R'=α-L-rhamnosyl

results in yields 0.5-2.25 g rhamnolipid/L using continuous culture conditions(19,20) and of up to 15 g/L using resting cells(18). Although rhamnolipids had been described as biosurfactants and emulsifiers, their use as a potential source of rhamnose had not been considered.

The organism studied in our laboratory(21,22) was a *Pseudomonas aeruginosa* isolate UI29791. This organism produced an emulsifier when grown on defined media in liquid culture. The production of rhamnolipid by *P. aeruginosa* was examined on medium containing glucose, corn oil, or glucose and corn oil as carbon sources. (Table 2). When glucose was replaced with corn oil as the sole carbon source, significantly increased levels of rhamnolipid was obtained. Part of this increase in rhamnolipid can be attributed to a higher cell mass; 2.3 times greater when corn oil was used as the carbon source as compared to glucose alone. Rhamnose levels, however were between 11 and 27 times greater when corn oil was used in place of glucose. Other vegetable oils including soybean oil, coconut oil, and cottonseed oil result in similar cell growth and rhamnolipid production. Levels of N,S,P,Mg,Ca,Fe and trace metals all had less effect on rhamnolipid production than did the choice of carbon source.

After the optimal nutritional requirements for rhamnolipid production by *P. aeruginosa* were defined, fermentor studies, 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) over 10 days gave a maximal cell density of 15 g/L on day 5 and a maximal rhamnolipid level on day 8 of 46 g/L. This level corresponds 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. A semicontinuous batch fermentation improved the overall volumetric productivity to 6.4 rhamnolipid/L-day. The maximum volumetric productivity, observed after the first media change and before maximum cell density was reached, was 10 g rhamnolipid/L-day.

At the completion of the fermentation cells were removed by centrifugation and the rhamnolipid was precipitated by acidifying the supernatant. The precipitate was recovered by centrifugation and hydrolyzed with sulfuric acid. The 3-hydroxydecanoic acid (the lipid portion of the hydrolysis mixture) was extracted with ethyl acetate, yielding a pure solution of rhamnose.

Comparison of Microbial Production of Rhamnose from Exopolysaccharides with its Production from Rhamnolipids

The mixture of rhamnolipids obtained (III) contains approximately 40 wt% rhamnose while the rhamnose content of the microbial exopolysaccharides varies between 12 and 55 wt%. *P. aeruginosa* yields 25 g rhamnose/L with a productivity of 15 mg rhamnose/g dry cell weight-h. The yields of rhamnose from *Klebsiella* and *Acinetobacter* were only 3.5-4.0 g rhamnose/L with productivities of 2.5-4.5 mg rhamnose/g dry cell weight-h.

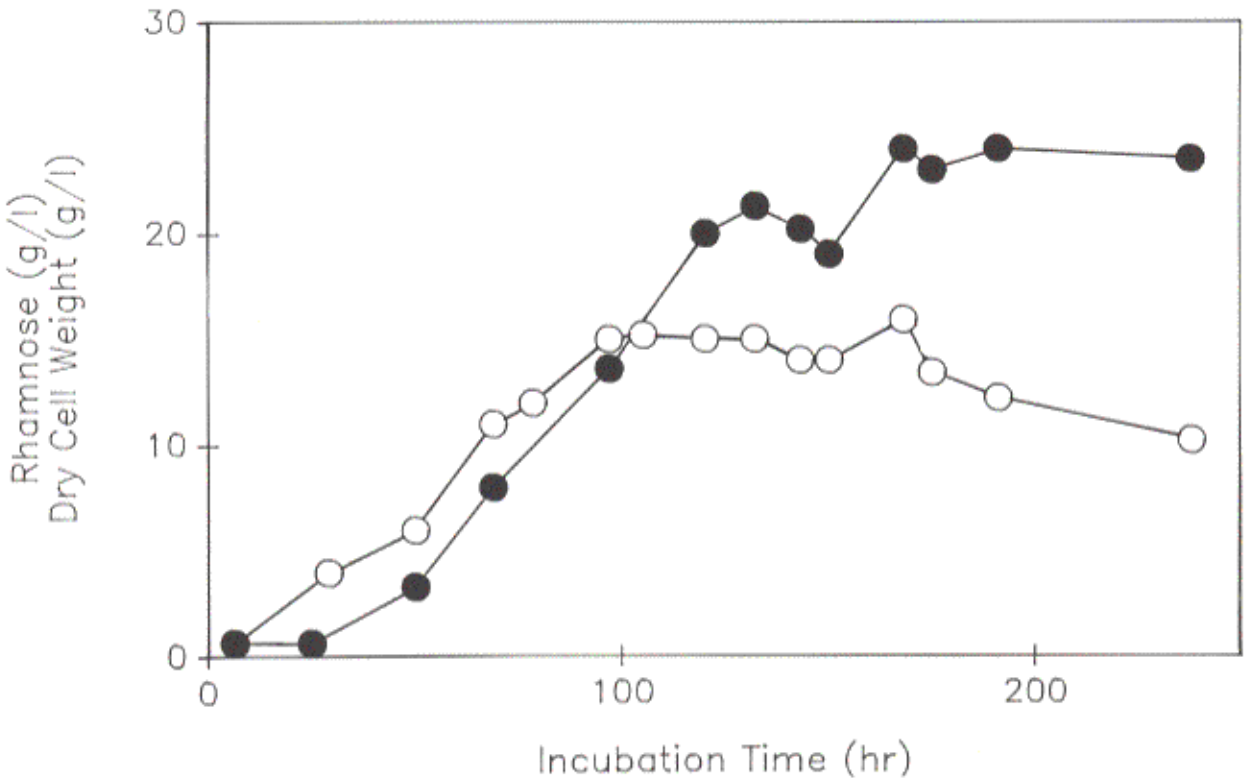


Fig. 2. Batch fermentation of *Pseudomonas aeruginosa*. Time (h) is plotted *vs.* ● dry cell weight and ○ rhamnose concentration (in g/L).

In addition to greatly increased yields and productivities, rhamnose preparation from rhamnolipid eliminates the problems associated with the exopolysaccharide source (discussed above) including high culture viscosity, recovery and purification problems. Finally, corn oil is a readily available, low cost, carbon source. The absence of a sugar-based carbon source in the growth medium also avoids potential sugar contamination of the rhamnose product.

Conclusions

This paper describes two methods by which an unusual deoxysugar having potential commercial value can be prepared using fermentation. Both methods represent improvements on the extraction of rhamnose containing compounds from plant materials. The preparation of sugars from microbial exopolysaccharides has a number of pitfalls, not least of which are associated with the physical properties of these polymers particularly their high molecular weight and high viscosity. The microbial preparation of sugars conjugated to fatty acids or possibly other low molecular weight carriers can reduce the problems of culture medium viscosity and product contamination with undesired sugars. The approaches discussed should be directly applicable to the microbial production of other high-value sugars(22).

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NOTICE TO SIM MEMBERS. . .

CHANGE IN FINANCIAL YEAR

At the 15 March 1991 SIM Board of Directors Meeting, the Board voted to change from a fiscal year of operation (1 July - 30 June), to a calendar year (1 January - 31 December).

The dues for 1991 will be prorated for a six month period (1 July - 31 December).

The 1992 full calendar year dues will be billed in mid October 1991.