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## Microbial production of biosurfactants

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### Abstract

Biosurfactants are those chemicals which are produced by microorganisms but which have both clearly defined hydrophilic and hydrophobic groups. They occur in nature in bacteria, yeasts, and fungi, and in particular in bacteria which grow on a water-immiscible substrate, using it as a food source. By evolution these bacteria have adapted themselves to feeding on these substrates by manufacturing and using a surface active product that helps the bacteria to adsorb, emulsify, wet, or disperse or solubilise the water immiscible material. The four main types of biosurfactant are: (1) glycolipids, (2) phospholipids, (3) lipoproteins or lipopeptides, (4) polymeric. By growth of the bacterium *Pseudomonas fluorescens* (NCIMB 11712) on virgin olive oil, production of a glycolipid in the form of a rhamnolipid is thought to have taken place. This biosurfactant group is based on the rhamnose structure, which is a methyl pentose monosaccharide. However, rhamnolipids can also be based on the disaccharide by condensing two moles of rhamnose together. The link to the hydrophobic group is by way of an acetal group; however, the 'lipid' part of the molecule contains ester and carboxyl groups. Biosurfactants over the years have found a great many uses in industry, for example, (1) oil recovery, (2) oil spill clean-up, (3) textiles, (4) pharmaceuticals, (5) cosmetics. By this programme of research, it is proposed to produce a biosurfactant for use in the detergents/cleaning materials industries — with the specific aim being to produce a biosurfactant which could be used in place of chemical surfactants in a detergent formulation for use in the household. Copyright © 1996 Elsevier Science B.V.

*Keywords:* Biosurfactant; Microbial; Rhamnolipid

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## 1. Introduction

In recent years detergent phosphates have been blamed for eutrophication in certain inland lakes and ponds — leading to the depletion of aquatic life in these areas. Oil spillages have destroyed marine life and the synthetic detergents used to clean up these spillages have often led to more destruction of the environment. From an environmental view point it is important that all substances released into the environment are biodegradable, firstly to assess their potential for causing environmental damage and secondly to safeguard against the possibility of future harm due to build-up in the environment. Micro-organisms, because of their large surface-to-volume ratio and diverse synthetic capabilities are promising candidates for widening the present range of surfactants. Biosurfactants are those chemicals which are produced by micro-organisms but which have both clearly defined hydrophobic and hydrophilic groups. They are produced by bacteria, yeasts and fungi (Table 1), and particularly in bacteria which are in a state of growth on a water-immiscible substrate which is a source of food for example crude oil spillage treated with selected microorganisms. By evolution, the bacteria have adapted themselves to feeding on water-immiscible materials by manufacturing and using a surface active product that helps the bacteria which are in the aqueous phase to adsorb, emulsify, wet, and disperse or solubilise the water-immiscible material.

The main types of biosurfactant are:

- (1) glycolipids,
- (2) phospholipids,
- (3) lipopeptides and lipoproteins,
- (4) polymeric.

Table 1  
Microbial biosurfactants and bioemulsifiers [11]

Microorganism	Biosurfactant/bioemulsifier
<i>Torulopsis</i> species	Sophorolipids
<i>Pseudomonas</i> species	Rhamnolipids
<i>Rhodococcus erythropolis</i>	Trehalose lipids
	Trehalose mycolates
	Sucrose and fructose lipids
<i>Rhodococcus</i> species H13-A	Trehalose lipids
<i>Candida</i> species	Mannosyl erythritol lipid
<i>Candida bogoriensis</i>	Sophorolipid
<i>Acinetobacter</i> species	Fatty acid, glycerides, emulsan
<i>Corynebacterium lepus</i>	Corynemycolic acids
<i>Candida petrophilum</i>	Peptidolipid
<i>Bacillus subtilis</i>	Cyclic lipopeptide
<i>Bacillus licheniformis</i>	Cyclic lipopeptide
<i>Candida tropicalis</i>	Mannan-fatty acid complex
<i>Corynebacterium hydrocarboclastus</i>	Proteo-lipid-carbohydrate complex

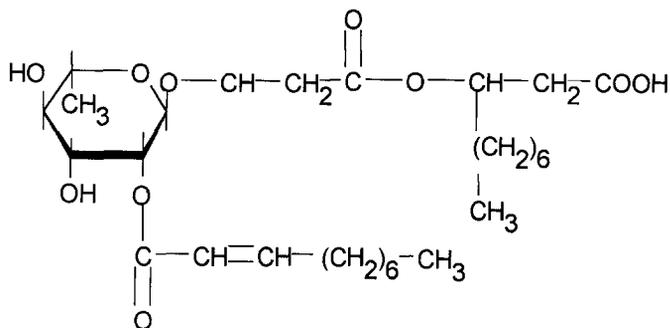


Fig. 1. A glycolipid produced by a *Pseudomonas* strain [10].

### 1.1. Glycolipids (Fig. 1)

These are compounds of a carbohydrate and a lipid; the linkage is by way of either an ether or an ester group. The main glycolipids which are found to occur and are most often investigated are:

- rhamnolipids,
- mycolates of mono, -di and -trisaccharides,
- sophorolipids.

### 1.2. Phospholipids

These are the esters formed between the alcohol groups on a lipid and a phosphate.

### 1.3. Lipopeptides and lipoproteins (Fig. 2)

These consist of a lipid attached to a polypeptide chain.

### 1.4. Polymeric

These are products again formed between saccharide units and fatty acid residues; but they are polymeric in nature.

Of the four types listed, it is the glycolipid and lipopeptide groups which are of particular interest, since the bacterial species used in this study i.e. *Pseudomonas fluorescens* (NCIMB 11712) is known to produce both types, especially the former.

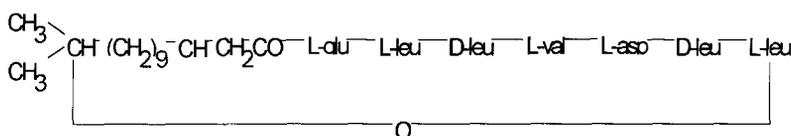


Fig. 2. A lipopeptide structure (surfactin) produced by *Bacillus subtilis* [10].

Table 2  
Levels of uronic acid present in oil phase of fermentation medium

Conc. of sodium nitrate solution (g/l)	Av. conc. of uronic acid present ( $\mu\text{mol/ml}$ )
1	0.3
2.5	0.22
2.5*	0.96
4	0.74
6	0.18

\*Indicates that solution has been made with tap water and not distilled water.

Several industrial applications of biosurfactants have been envisioned. However, at present the greatest potential use is by the oil industry. Compared to chemical surfactants in an oil-water emulsion, the requirements of a biosurfactant are much less and they are more selective. Probably the most important advantage of biosurfactants over chemical surfactants is their ecological acceptability. Many chemically synthesised surfactants cause ecological problems owing to their resistance to degradation, toxicity, and accumulation in natural ecosystems [1]. On the other hand, biosurfactants are biodegradable [2].

However, the main limiting factor is the economics of large-scale production of biosurfactants [3]; the main drawbacks being:

- (1) poor yields from raw substrate materials;
- (2) large capital investment;
- (3) reactions are carried out in dilute solution so there is poor volume efficiency for the plant;
- (4) need for sterilisation;
- (5) problems in the control of the process, for example, foaming;
- (6) problems in product recovery and purification;
- (7) difficulties in analysing the finished products chemically due to their complex nature.

This research work concentrated on the potential of a selected bacterium *Pseudomonas fluorescens* to produce a biosurfactant or biosurfactants for both virgin olive oil and chip-pan oil substrates. *Pseudomonas fluorescens* (NCIMB11712) was incubated with olive oil and a sodium nitrate solution and

Table 3  
Levels of uronic acid present in aqueous phase of fermentation medium

Conc. of sodium nitrate solution (g/l)	Av. conc. of uronic acid present ( $\mu\text{mol/ml}$ )
1	0
2.5	0.042
2.5*	0.34
4	0.26
6	0.032

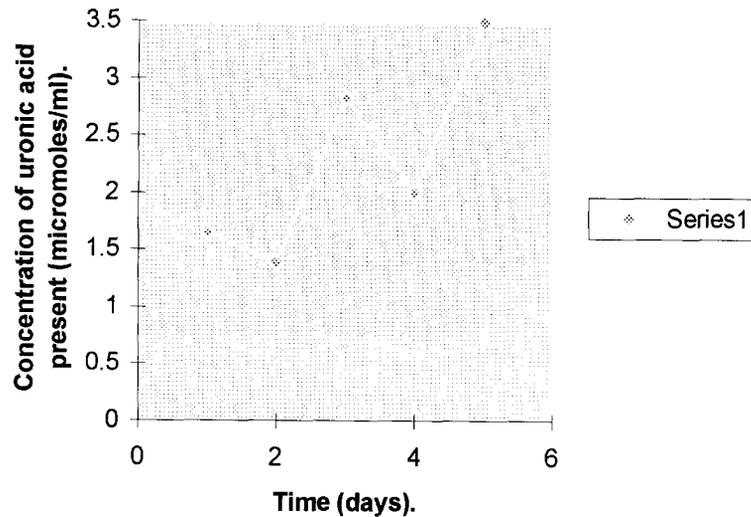


Fig. 3. Variation in the concentration of uronic acid present with time [ $\text{NaNO}_3$  conc. = 2.5 g/l\*].

although it was initially thought that the biosurfactant produced belonged to the glycolipid group of biosurfactants, subsequent analysis has led to the conclusion that the biosurfactant produced under these conditions belongs in fact to the lipopeptide group of biosurfactants, and is a carbohydrate-protein-lipid complex (Fig. 2).

## 2. Experimental

### 2.1. Production of biosurfactants

Culture conditions and subsequent analysis were as outlined in the paper by Mercade et al. [4]. Olive oil was chosen as one of the substrates due to the waste produced during its processing [4]. Initially microbial growth and biosurfactant production experiments were carried out in 500 ml shake flasks containing 250 ml of medium, i.e. 225 ml of sodium nitrate solution of the following concentrations: 1, 2.5, 4 and 6 g/l (using distilled water for dilution), and 2.5 g/l using tap water and 25 ml of olive oil. A cell suspension from 24 h nutrient agar cultures was used as inoculum and flasks were incubated at 30°C in a New Brunswick G100 gyratory-incubator shaker at 200 rpm. Cells were separated from the culture broth by centrifugation at  $8000 \times g$  for 15 min.

## 2.2. Chemical analysis of biosurfactants

Isolation of surface active compounds from culture supernatant was performed using conventional methods [5]. The wet cell paste containing about 40–50 mg of cells (dry weight) was diluted to 1 ml with water and to the suspension 3.75 ml of methanol/chloroform (2:1 v/v) was added. The mixture was shaken and left at room temperature for several hours with intermittent shaking. After centrifugation, the supernatant extract was decanted and to the residue 4.75 ml of methanol/chloroform/water (2:1:0.8) was added; the mixture was then shaken and centrifuged. To the combined supernatant extracts, 2.5 ml of both chloroform and water were added and the mixture centrifuged. The lower chloroform phase was withdrawn, diluted with benzene (to aid removal of traces of water) and brought to dryness in a rotary evaporator (30–35°C). The lipid residue was immediately dissolved in chloroform/methanol (1:1) and the solution centrifuged and made to a known volume with chloroform.

## 2.3. Rhamnolipid concentration

This was estimated by colorimetric determination of rhamnose concentration by the method of Chandrasekaran and Bemiller [6]

## 2.4. Uronic acid

Samples were heated in concentrated sulphuric acid containing borate and then reacted with carbazole.

### 2.4.1. Reagents

- (1) Borate-sulphuric acid: 3.82 g of sodium borate was dissolved in 10 ml of hot water and 390 ml of well-cooled concentrated sulphuric acid was added by stirring with a glass rod. This reagent was stored in a refrigerator in a glass bottle.
- (2) 0.2% carbazole: 100 mg of carbazole was dissolved in 50 ml of absolute ethanol and stored in a refrigerator in a brown glass bottle.
- (3) Standard glucuronolactone: 17.6 mg of D-glucuronolactone was dissolved in 100 ml of deionized water (1  $\mu\text{mol/ml}$ ) just before use.

### 2.4.2. Procedure

Aliquots of the standard solution of glucuronolactone 0 to 0.2 ml (by 0.02 ml increments) were transferred by pipette to test tubes. After bringing the volume in each tube to 0.5 ml with water, 3 ml of cold borate-sulphuric reagent was added with immediate mixing. The tubes were heated in a boiling water bath for 20 min. After cooling to 0°C, 0.1 ml of 0.2% carbazole solution were added. The solution was shaken well and heated again in the boiling water bath for 10 min. After cooling at room temperature for 15 min the colour was read at 530 nm against the

blank. Three different aliquots of the sample were subjected to this reaction to determine the amount of uronic acid present.

## 2.5. Hexosamines

A sample containing 0.3–0.5  $\mu\text{mol}$  of uronic acid in 0.2 ml of water was placed in a small ampoule (2 ml capacity), mixed with 0.2 ml of concentrated hydrochloric acid, sealed and heated in a thermoblock at 100°C for 4 h. After hydrolysis, the ampoule was opened and placed in a vacuum dessicator containing sodium hydroxide pellets in a beaker. After complete drying, the residue in the ampoule was dissolved in 0.3 ml of water.

### 2.5.1. Procedure

Hexosamines were reacted with an alkaline solution of 2,4-pentanedione to form chromogens, which yield colour with *N,N*-dimethyl-*p*-aminobenzaldehyde in acid. Both glucosamine and galactosamine form chromogens when this reaction is done at 100°C, whereas only galactosamine forms chromogens when the reaction is done at 0°C. Hence, the method can be used to give the glucosamine/ galactosamine ratio.

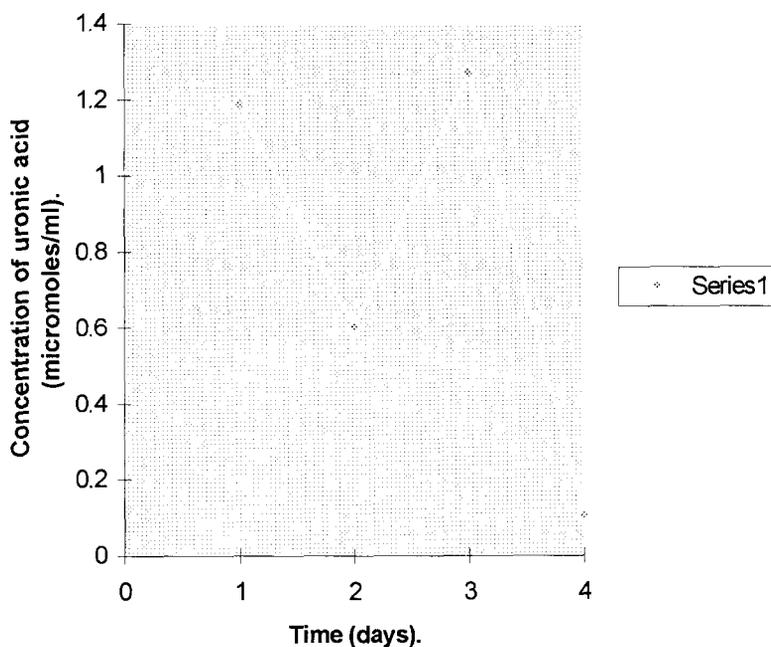


Fig. 4. Variation in the concentration of uronic acid present with time [ $\text{NaNO}_3$  conc. = 2.5 g/l].

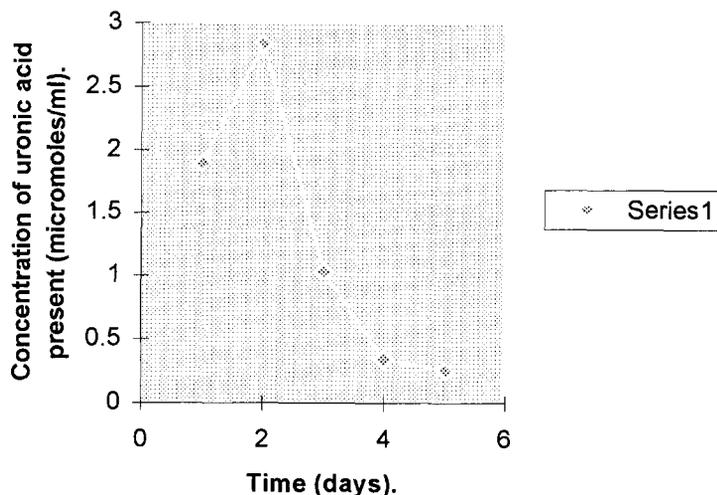


Fig. 5. Variation in the concentration of uronic acid present with time for a control run [not inoculated,  $\text{NaNO}_3$  conc. = 2.5 g/l].

#### 2.5.2. Reagents

- (1) Alkaline 2,4-pentanedione: 0.15 ml of 2,4-pentanedione was diluted to 5 ml with 0.7 M sodium carbonate. This reagent was prepared just before use.
- (2) Ehrlich reagent: 100 mg of *N,N*-dimethyl-*p*-aminobenzaldehyde is added to 3.2 ml of 60% perchloric acid and then made up to 10 ml with 95% ethanol. This reagent must be freshly prepared before use.
- (3) 90% ethanol.
- (4) Hexosamine hydrochloride solution, 2  $\mu\text{mol/ml}$ .

#### 2.5.3. Total hexosamine

Aliquots of the standard solution of hexosamine hydrochloride (0 to 0.1 ml by 0.01 ml increments) were transferred by pipette to screw top tubes. After making up the solutions to 0.1 ml with water, 0.025 ml of 1.5 M hydrochloric acid was added with thorough mixing. The tubes were capped and heated in a boiling water bath for 20 min, then cooled to approximately 20°C by immersion in cold water. Then 2 ml of 90% ethanol and 0.5 ml of Ehrlich reagent were added with thorough mixing, and the tubes allowed to stand at 20°C–25°C for 1 h. The colour was read at 535 nm against the blank.

#### 2.5.4. Galactosamine

Standards of galactosamine were prepared as for total hexosamine; several blanks containing 0.2  $\mu\text{mol}$  or more of standard glucosamine were also prepared. The tubes were placed in an ice/salt bath at 0°C and to these were added. First, 0.25 ml of 1.5 M hydrochloric acid were added (0°C) and then 0.25 ml of pentanedione

reagent (0°C). The tubes were capped, the contents were mixed thoroughly, and samples kept at 0°C for 18 h. After incubation, 2 ml of 90% ethanol and 0.5 ml of Ehrlich reagent were added; the contents were mixed, and the samples heated in a water bath at 70°C for 1 h. These were then kept at 20°C–25°C for 1 h and the absorbance of each recorded at 525 nm.

It must be noted that although all the tests included in this paper were used, only that for uronic acid proved positive and it was decided at this point to use only uronic acid determination as a marker for the production of a rhamnolipid by the bacteria under the conditions employed for each particular fermentation.

### 3. Rapid method for monitoring maximum biosurfactant production obtained by acetone precipitation [7]

#### 3.1. Isolation of the biosurfactant

The organisms were cultivated as before in a medium of olive oil and a sodium nitrate solution, the culture broth was centrifuged at 10 000 rpm for 30 min at 4°C, and the clear supernatant recentrifuged at 10 000 rpm for 30 min at 4°C, after which the cell free supernatant was treated with 3 volumes of chilled acetone. The precipitate was collected by centrifugation at 5000 rpm for 10 min and dried under a current of air.

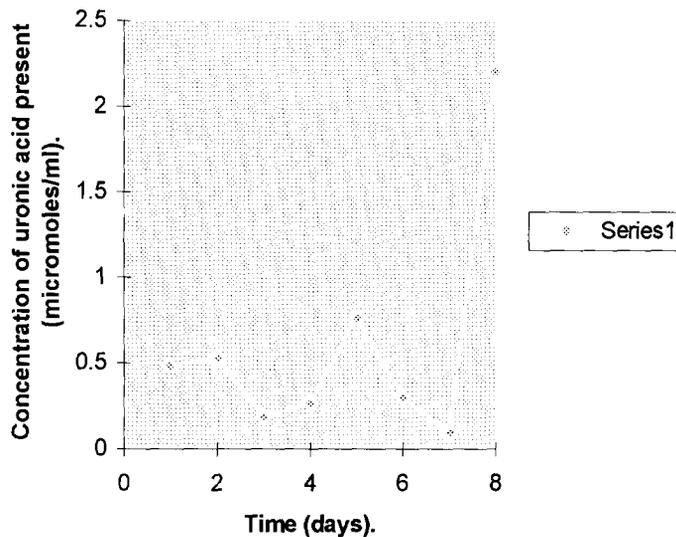


Fig. 6. Variation in the concentration of uronic acid present with time [ $\text{NaNO}_3$  conc. = 2.5 g/l\*].

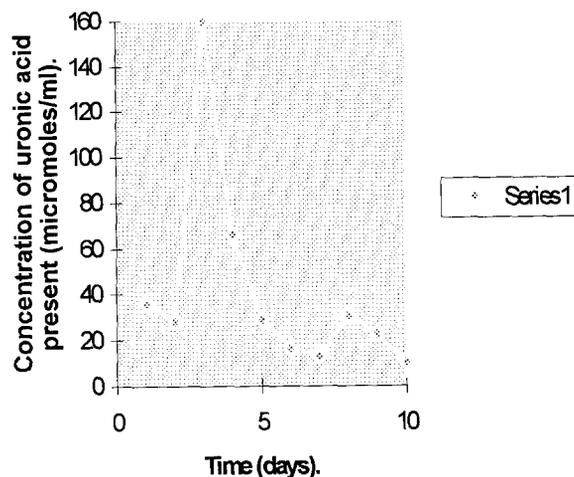


Fig. 7. Variation in the concentration of uronic acid present with time [ $\text{NaNO}_3$  conc. = 2.5 g/l; oil/aqueous phase ratio, 1:3 v/v].

### 3.2. Estimation of biomass

Discarded cells from the isolation of the biosurfactant process were centrifuged at 12000 rpm for 15 min and extracted with a mixture of acetone/hexane (3:1) to remove adhering hydrocarbon. This was followed by centrifugation with hexane and drying overnight to obtain dry biomass.

## 4. Chemical analysis

### 4.1. Protein estimation

Protein in the isolated biosurfactant was estimated by the Pierce BCA method; this involves the use of a sensitive reagent, bicinchonic acid (BCA) for the spectrophotometric determination of protein concentration in solutions. It combines the reaction of protein with  $\text{Cu}^{2+}$  in an alkaline medium (yielding  $\text{Cu}^+$ ) with BCA. A purple reaction product results whenever two molecules of BCA interact with one of  $\text{Cu}^+$ . This complex is water soluble and exhibits a strong absorbance at 562 nm.

Three protocols may be used:

Standard protocol: 37°C for 30 min

Room temperature protocol: room temperature for 2 h

Enhanced protocol: 60°C for 30 min.

#### 4.1.1. Working reagent

One part reagent B was added to 50 parts reagent A with thorough mixing, this is stable for 1 day at room temperature. Reagent A = sodium carbonate

Reagent A:

sodium carbonate

sodium bicarbonate

BCA detection reagent

sodium tartrate in 0.2 N NaOH

Reagent B:

4% copper sulphate solution

#### 4.1.2. Method

First, 0.1 ml of each standard or unknown protein sample were pipetted into appropriately labelled test tubes, water being used as a blank. To each tube 2 ml of working reagent was added and mixed well, the tubes were incubated at the selected protocol after which they were all allowed to cool to room temperature, and the absorbance was read at 562 nm, that for the blank solution being subtracted from the values determined.

Standards consisted of: 0, 50, 100, 150, 200 and 250  $\mu\text{g/ml}$  of B.S.A. protein standard as provided.

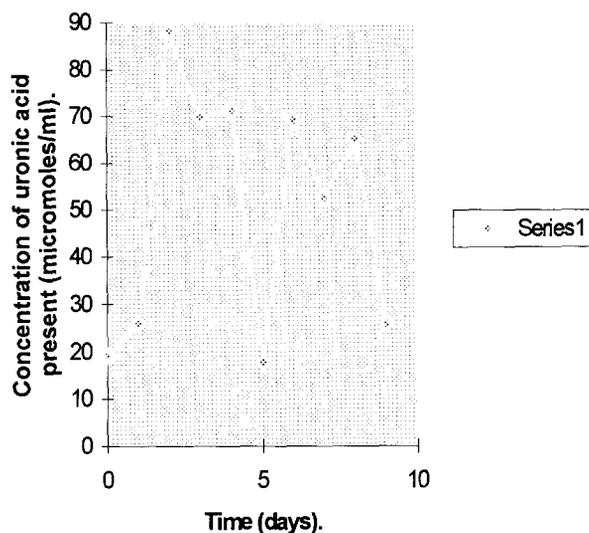


Fig. 8. Variation in the concentration of uronic acid present with time [ $\text{NaNO}_3$  conc. = 2.5 g/l\*; oil/aqueous phase ration, 1:3 v/v].

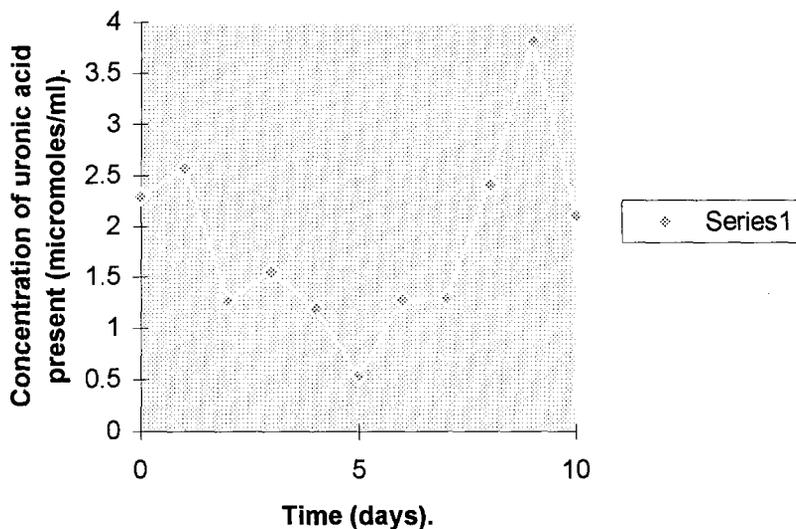


Fig. 9. Variation in the concentration of uronic acid present with time [ $\text{NaNO}_3$  conc. = 2.5 g/l\*; oil/aqueous phase ratio, 1:1 v/v].

#### 4.2. Carbohydrate estimation [8]

##### 4.2.1. Anthrone reagent

Sulphuric acid (66%): 340 ml distilled water and 660 ml of concentrated sulphuric acid (s.g. 1.84). First, 500 mg of recrystallised anthrone and 10 g thiourea were added to 1 l of 66% sulphuric acid and the mixture warmed to 80–90°C, shaking the flask occasionally. This was stable for 2 weeks in a refrigerator.

##### 4.2.2. Standard glucose solution

Stock solution: glucose powder was dried in an oven at 60–70°C, after which 100 mg were dissolved in 100 ml of saturated benzoic acid solution.

Working standard: 10 ml of stock solution were transferred to a 100 ml volumetric flask and the volume made up with saturated benzoic acid solution. Then, 1 ml of this solution containing 0.1 mg of glucose was used as a standard.

Deproteinising reagents: 5% trichloroacetic acid.

##### 4.2.3. Method

The sample was deproteinised with 5% trichloroacetic acid, dilution being 1:10. To three appropriately labelled test tubes were added the following: 1 ml of sample, 1 ml of glucose standard, and 1 ml of distilled water. To each, 10 ml of anthrone reagent were added with thorough mixing the tubes were stoppered and placed in water at room temperature for 3–5 min, after which they were placed in a boiling water bath for 15 min with light being excluded. The tubes were then removed to water at room temperature again and allowed to stand for a further 20–30 min. The absorbance of the samples were then measured at 620 nm.

The following equation was used to calculate the amount of carbohydrate present:  $DU/DS \times 0.1 \times \text{dilution of sample} \times 100 = \text{mg glucose/100 ml}$ , where

DU: optical density of sample

DS: optical density of standard

0.1: mg glucose/ml standard solution.

#### 4.3. Lipid estimation

To determine the lipid content, 0.5 g of the isolated material was extracted with 25 ml of diethyl ether on a water bath at 50°C for 30 min. The ether extract was dried using anhydrous  $\text{Na}_2\text{SO}_4$  and the lipid content determined by gravimetric estimation.

#### 4.4. Measurement of emulsification activity

To estimate the emulsification activity, 6 ml of *n*-dodecane was added to 4 ml of the culture broth in a graduated tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h. The emulsification index,  $E_{24}$  (%) was calculated by dividing the height of the emulsion layer by the total height of the mixture and multiplying it by 100.

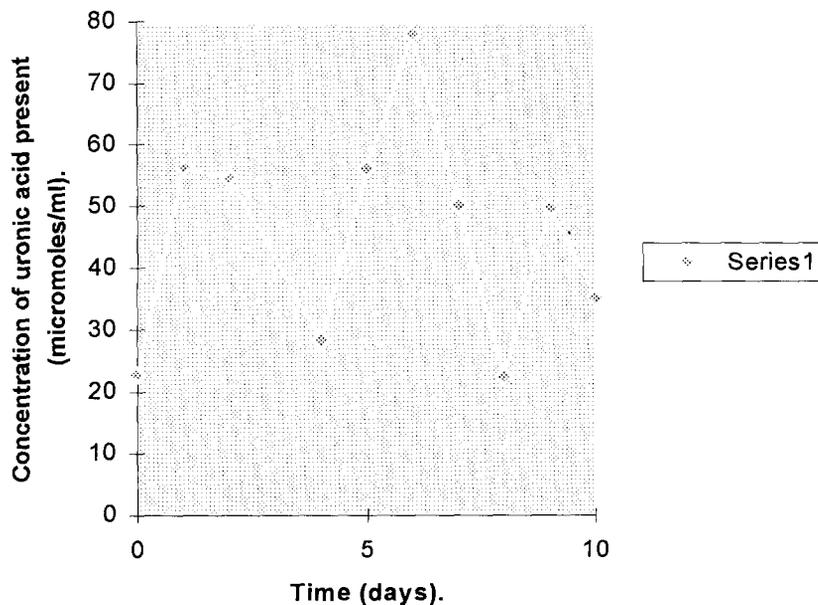


Fig. 10. Variation in the concentration of uronic acid present with time [ $\text{NaNO}_3$  conc. = 2.5 g/l; oil/aqueous phase ratio, 1:1 v/v].

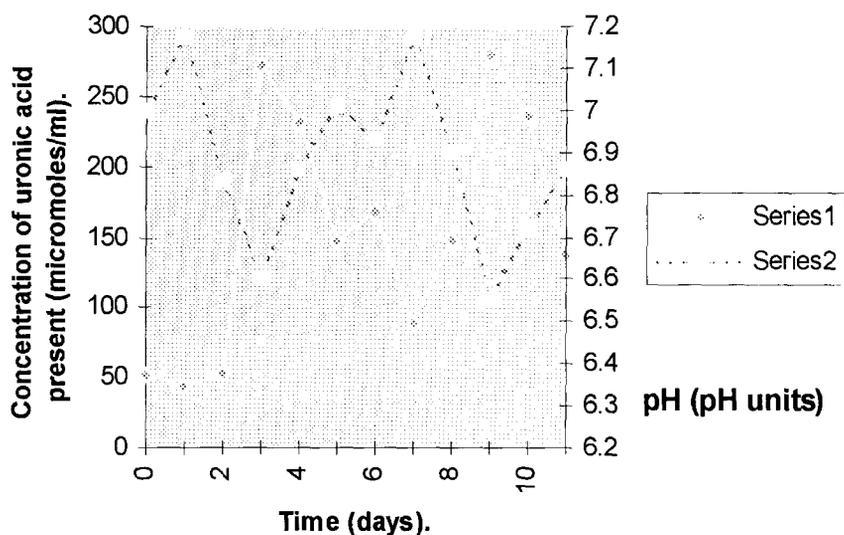


Fig. 11. Variation in the concentration of uronic acid present and the pH with time for a control run [run inoculated,  $\text{NaNO}_3$  conc. = 2.5 g/l; oil/aqueous phase ratio, 1:1 v/v].

For measurement of the emulsification activity of the acetone precipitated biosurfactant 4 ml of the biosurfactant solution (1 mg/ml) in water was taken. To this, 6 ml of *n*-dodecane was added and the emulsification activity was determined as above.

#### 4.5. Surface tension measurement

This was measured by a 'White' surface and interfacial tension torsion balance, model OS.

### 5. Discussion and conclusions

It is thought that the bacteria in the fermentation medium metabolise the oil to produce glucose, the terminal  $\text{CH}_2\text{OH}$  group on the glucose molecule is then oxidised to a carboxyl group, producing a uronic acid — glucuronic acid [9]. Thus, uronic acid determination was used as a marker for biosurfactant production and as such was also used to determine the optimum concentration of sodium nitrate solution in the fermentation medium, Tables 2 and 3 show the levels of uronic acid for each concentration of sodium nitrate solution used, as can be seen from the results the maximum concentration of uronic acid present was for a 2.5 g/l\* (where \* indicates that the solution was made with tap water and not distilled water) solution. It was decided then to use this sodium nitrate solution in the fermentation medium, and also to use a solution of the same concentration but one which was

made with distilled water to run in parallel with these fermentations as a comparison.

Initially analyses of all incubations involved determination of uronic acid as an indicator of biosurfactant production by *Pseudomonas fluorescens*. The latter is well known as both a biosurfactant producer and a secretor of specific lipases for the hydrolysis of fats. As oils (in this case virgin olive oil) consist virtually of fat only, then either or both production of a biosurfactant or lipases would be possible on incubation with *Pseudomonas fluorescens*.

As can be seen from Figs. 3–13, depicting uronic acid levels in the incubation media, the levels in some cases are very high, then it can be fairly sure that a biosurfactant is being produced. No attempt was made to assess for lipase production in this work since it is the primary desire to achieve maximum biosurfactant production.

However evidence at a later stage emerged that protein was present. Hence, it is now assumed that a rhamnolipid and a proteinaceous material are both present but at different stages.

With the levels of uronic acid, as detected in the samples chosen, varying from high to low and then returning to high again, with the trend being repeated, it is thought that for biosurfactant production to continue to help achieve maximum emulsification then a rhamnolipid is produced followed by either a second, lipoprotein biosurfactant or enzyme (lipase) activity. The latter has yet to be proven.

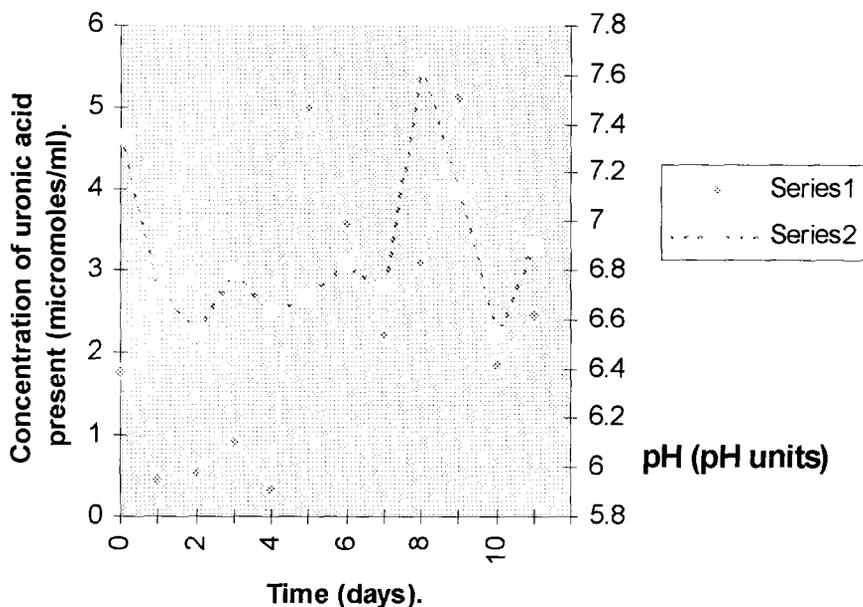


Fig. 12. Variation in the concentration of uronic acid present and the pH with time for a run using chip-pan oil [ $\text{NaNO}_3$  conc. = 2.5 g/l; oil/aqueous phase ratio, 1:1 v/v].

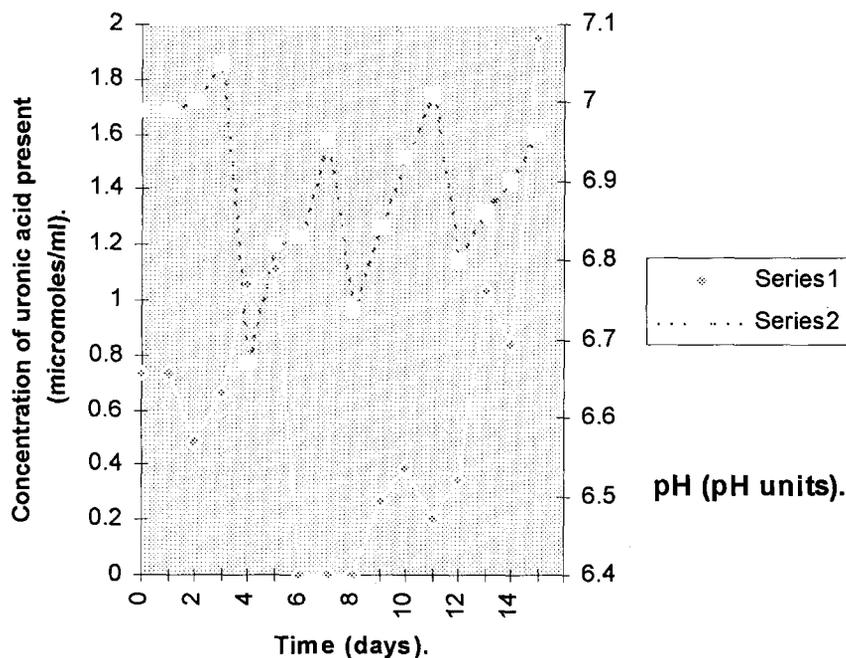


Fig. 13. Variation in the concentration of uronic acid present and the pH with time [ $\text{NaNO}_3$  conc. = 2.5 g/l\*; oil/aqueous phase ratio, 1:3 v/v].

This work on virgin olive oil has been used to, in the first instance, induce *Pseudomonas fluorescens* to produce a biosurfactant of the rhamnolipid class, and the presence of uronic acid is indicative of this. The presence of the proteinaceous material and its exact role has yet to be clarified. The work is now to be extended to waste chip-pan oil using a microprocessor-controlled bioreactor with the parameters (pH, dissolved oxygen, temperature and agitation rate) to be optimised.

Analysis of samples taken from a fermentation using olive oil and a 2.5 g/l\* solution of sodium nitrate as the fermentation medium with an oil to aqueous phase ratio of 1:3 yielded the following results (see also Fig. 13):

Protein concentration: 0.908 g/l

Carbohydrate concentration: 0.022 mg glucose/l

Lipid concentration: approx. 0.1 g/l

Emulsification activity:  $E_{24}$  (%) precipitate = 6.15%

CHN analysis:

C:28.15%

H:4.10%

N:8.83%

Surface tension: 0.033  $\text{Nm}^2$

Biomass: 0.8 g/l

Analysis of samples taken from a fermentation using olive oil and a 2.5 g/l solution of sodium nitrate as the fermentation medium with an oil to aqueous phase ratio of 1:1 yielded the following results (see also Fig. 10):

Protein concentration: 0.011 g/l

Carbohydrate concentration: 0.00008 mg glucose/l

Lipid concentration: approx. 0.3 g/l

Emulsification activity:

E<sub>24</sub> (%) broth = 0.083%

E<sub>24</sub> (%) precipitate = 1.67%

CHN analysis:

C: 11.17%

H: 2.69%

N: 11.84%

Surface tension measurement: 0.032 Nm<sup>2</sup>

Biomass: 0.1 g/l

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