Exopolysaccharide Production by *Pseudomonas* NCIB11264 Grown in Continuous Culture

By A. G. WILLIAMS* AND J. W. T. WIMPENNY

Department of Microbiology, University College, Cardiff CF2 1TA

(Received 22 June 1977; revised 24 August 1977)

Exopolysaccharide formation by *Pseudomonas* NCIB11264 in a single-stage continuous culture was maximal under nitrogen limitation with excess carbohydrate substrate at 30 ± 1 °C and pH 7.0 ± 0.1 . Polysaccharide production was not enhanced by phosphate limitation but was dependent on the dilution rate. Steady states were maintained for up to 500 h without deterioration of the culture or the development of mutant strains. The efficiency of conversion of the glucose substrate utilized into exopolysaccharide by the chemostat cultures was as high as 73 %.

INTRODUCTION

Pseudomonas NCIB11264 forms an extracellular acylated slime polysaccharide in which the monosaccharides glucose, galactose, rhamnose and mannose have been detected (Williams, Wimpenny & Lawson, 1973). Preliminary batch fermentation studies indicated that the exopolysaccharide is synthesized over a wide range of cultural conditions, although the amount of polymer produced is influenced by medium composition and environmental factors (Williams & Wimpenny, 1977). The effect of growth conditions on microbial exopolysaccharide production has, in the past, been mainly determined by plate and small-scale batch culture techniques, whilst the obvious potential of continuous culture both as a research and production tool has remained largely unexploited. However, the production of the exopolysaccharide xanthan gum by *Xanthomonas campestris* has been studied not only in batch cultures at both the laboratory (Lilly, Wilson & Leach, 1958) and pilot plant level (Rogovin, Anderson & Cadmus, 1961) but also in single-stage continuous (chemostat) fermentations (Lindblom & Patton, 1967; Rogovin, 1969). The kinetics of the xanthan biopolymer fermentation, determined in batch culture experiments (Moraine & Rogovin, 1966, 1973), were shown to be applicable to continuous fermentations in which the xanthan production rate was a function of the dilution rate and pH (Silman & Rogovin, 1970, 1972).

In this paper, we report the effects of growth parameters on exopolysaccharide formation by *Pseudomonas* NCIB11264 in a single-stage continuous fermenter. A preliminary report of some of these findings has already been published (Williams & Wimpenny, 1976).

METHODS

Maintenance of culture and preparation of inoculum. *Pseudomonas* NCIB11264 was maintained on nutrient agar slopes supplemented with glucose (2 %, w/v) and subcultured at monthly intervals with overnight incubation at 30 °C followed by storage at 4 °C. Inocula (200 ml) were prepared from a single slant in a chemically defined medium containing glucose (2 %, w/v) as carbon source and the following components (mg l^{-1}): NH_4Cl (2660), KH_2PO_4 (5440), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (60), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (6), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.4), and

* Present address: Department of Biochemistry, Hannah Research Institute, Ayr, Scotland KA6 5HL.

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.6), buffered to pH 7.0. A portion (20 ml) of the overnight culture (gyratory incubation, 120 rev. min^{-1} , 30 °C) was inoculated into the chemostat containing 500 ml medium. After inoculation the culture was established by batch growth for 24 h before the addition of medium was commenced. Steady states were allowed to establish for at least 72 h and all samples were subsequently removed directly from the fermentation vessel.

Continuous fermentation. The 1 l continuous culture system used was based on the 'Porton-type' chemostat (Evans, Herbert & Tempest, 1970), and included facilities for the control of pH, temperature, gas flow rates, impeller speed and foaming (Williams, 1974). The fermentation vessel, medium reservoirs (3×20 l) and all other in-line vessels (containing acid/alkali, antifoam, glucose component of the defined medium) were fully assembled prior to autoclaving (121 °C, 60 min). The medium was pumped into the growth vessel with an LKB Varioperpex 12000 peristaltic pump, and the effluent was discharged continuously through an overflow weir, set at the 550 ml level, into a sterile product collection unit. As the exopolysaccharide produced prevented foaming, antifoam was not added.

Determination of the oxygen transfer characteristics of the fermentation vessel. In the presence of catalytic amounts of copper ions, sulphite is rapidly oxidized to sulphate by dissolved oxygen, at a rate equal to that of oxygen solution (i.e. transfer). The iodometric method used was that of Cooper, Fernstrom & Miller (1944). The completely assembled nitrogen-sparged fermentation vessel was filled to its working capacity with 0.25 M-sodium sulphite, and after 10 min equilibration to 30 °C the gas flow rates were adjusted and the copper sulphate catalyst (0.1 mg) was added. Sulphite samples (10 ml) were removed at intervals and transferred to a titration flask containing 0.1 M-iodine solution (25 ml) and 2 M-HCl (5 ml). The residual sulphite was oxidized by the iodine and the unused iodine was titrated against 0.1 M-sodium thiosulphate with a starch indicator.

Analytical methods. The ammonia-nitrogen and phosphate contents of the growth medium and effluent liquor were estimated colorimetrically using the methods described by Solorzano (1969) and Fiske & Subbarow (1925) respectively; the glucose concentration was measured enzymically with glucose oxidase (Sigma). The total cell protein was determined in washed suspensions by the method of Lowry *et al.* (1951), after cell lysis with an equal volume of 1 M-NaOH (5 min, 100 °C). Total cell carbohydrate was determined by the phenol- H_2SO_4 reaction (Dubois *et al.*, 1956) with a glucose standard. Exopolysaccharide in cell-free culture supernatants was estimated by two of the following methods described by Williams & Wimpenny (1977): (i) colorimetrically, using the phenol- H_2SO_4 reaction; (ii) by viscometry, with a modified Zimm-Crothers viscometer (55 mA, 25 ± 0.2 °C); (iii) gravimetrically, after precipitation with propan-2-ol.

The recovery and preparation of polysaccharide samples for analysis and the techniques used in the identification of the monosaccharide components of the exopolymer and for the determination of hexose, deoxyhexose, acetate and pyruvic acid have been described (Williams & Wimpenny, 1977).

Viscosity measurement. Culture viscosity was measured at 25 ± 0.2 °C with an applied rate of shear of 437 s^{-1} using a Haake viscometer (Haake Instruments, Berlin) fitted with the NV rotary assembly (Williams, 1974). The exopolysaccharide exhibits pseudoplastic, but not thixotropic, rheological characteristics (Williams, 1974) and thus the apparent viscosity figures quoted for the fermentation liquor are shear-rate dependent.

RESULTS

Oxygen transfer characteristics of the system

The oxygen transfer characteristics of the fermenter were determined so that impeller speed and gas flow rates could be selected to ensure efficient aeration of the culture in the presence of exopolysaccharide. The effect of increasing impeller speed on oxygen solution at constant gas flow rate and temperature, and the uptake of oxygen from oxygen/nitrogen mixtures at constant impeller speed and temperature were examined. In both experiments, the total volume of gas passing through the fermenter was 500 ml min^{-1} and the temperature was 30 ± 1 °C. The oxygen uptake rate increased linearly with impeller speed from 500 to 1200 rev. min^{-1} (Fig. 1); it was also directly proportional to the partial pressure of oxygen in the inflowing gas mixture of constant volume and flow rate at a constant impeller speed (Fig. 1).

At a constant motor output the impeller speed decreased with increasing culture viscosity and the linear relationship between oxygen uptake rate and impeller speed was restricted to a much narrower range of impeller speeds (750 to 900 rev. min^{-1}) in the presence of low levels of exopolysaccharide (2.5 mg ml^{-1}) (Fig. 1). At impeller speeds of 700 to 1000 rev. min^{-1} the reduction in oxygen uptake rate was 10 % at a polysaccharide concentration of

Fig. 1. Oxygen uptake rates at various impeller speeds in the absence (□) or presence (●) of exopolysaccharide (2.5 mg ml^{-1}) under otherwise identical conditions (gas flow rate 500 ml min^{-1} , 30°C). Also shown is the oxygen uptake-flow rate relationship (■) (impeller speed $850 \text{ rev. min}^{-1}$, 30°C): the total gas flow was maintained at 500 ml min^{-1} in which O_2 was varied from 50 to 500 ml min^{-1} .

Fig. 2. Steady-state exopolysaccharide production by *Pseudomonas* NCIB11264 showing variations in E_{520} (○), exopolysaccharide concentration (Δ), glucose conversion efficiency (▲) and glucose concentration (□). The culture was established for 24 h prior to the metered addition of medium started at the time indicated by the arrow.

2.5 mg ml^{-1} , but became more pronounced as the impeller speed increased (20% at $1270 \text{ rev. min}^{-1}$). Thus, in the following series of experiments, the rate of aeration was maintained at 500 ml min^{-1} with an impeller speed of $900 \pm 10 \text{ rev. min}^{-1}$.

Exopolysaccharide production in continuous culture

Steady-state exopolysaccharide formation was examined under conditions of nitrogen limitation at $\text{pH } 7 \pm 0.1$ and $30 \pm 1^\circ \text{C}$, at a dilution rate of 0.08 h^{-1} for 500 h (Fig. 2). Steady-state values for exopolysaccharide levels and the efficiency of conversion of the glucose utilized into the exopolymer remained constant. There was no evidence of cultural deterioration or the development of mutant strains.

Polysaccharide samples analysed were of constant composition (Table 1) and solutions of representative polymer samples ($100 \mu\text{g ml}^{-1}$) had similar relative viscosities (1.70 ± 0.05) when measured at 25°C using a modified Zimm-Crothers rotating cylinder viscometer (Williams, 1969) with an applied current of 55 mA.

Fig. 3. Effect of dilution rate on steady-state exopolysaccharide production (Δ), glucose conversion efficiency ($\blacktriangle$), total cell population, estimated as E_{520} ($\circ$) and culture protein ($\bullet$), and concentration of NH_4Cl as limiting nutrient ($\blacksquare$).

Fig. 4. Effect of temperature on steady-state exopolysaccharide production (Δ), glucose conversion efficiency ($\blacktriangle$), E_{520} ($\circ$), total culture protein ($\bullet$) and culture viscosity at 25 $^{\circ}\text{C}$ (rate of shear 437 s^{-1}) ($\blacksquare$).

Effect of growth conditions on exopolysaccharide formation

Dilution rate. The effect of dilution rate ($D = 0.023$ to 0.23 h^{-1}) on exopolysaccharide production was determined in nitrogen-limited cultures at 30 $^{\circ}\text{C}$ and pH 7. The amount of polysaccharide produced was related to the dilution rate and decreased with increasing dilution rate, although the steady-state cell population remained relatively constant over the whole range of dilution rates (Fig. 3). The maximum yield of polysaccharide, from the glucose utilized, was 73 %. The composition of the exopolysaccharide produced at various dilution rates in the chemostat was not significantly different from that produced in batch culture (Table 1).

Table 1. *Exopolysaccharide formation by cells grown under various conditions in a chemostat*

Polysaccharide samples were prepared and analysed as described by Williams & Wimpenny (1977). The deoxyhexose content was less than 10 % in all samples, after correction for the hexose interference in the assay system.

Experimental variable	Steady-state conditions under which exopolysaccharide formed						Composition of exopolysaccharide (% w/w)		
	<i>D</i> (h ⁻¹)	pH	Temp. (°C)	Glucose concn (mg ml ⁻¹)	NH ₄ Cl concn (μg ml ⁻¹)	KH ₂ PO ₄ (mg l ⁻¹)	Hexose	Acetate	Pyruvate
Dilution rate (<i>D</i>)	0.023	7.0	30.0	13.8	1.1	ND	74.1	3.6	8.2
	0.038			14.9	2.7		71.6	3.5	6.9
	0.057			16.4	1.9		73.8	3.6	7.0
	0.176			19.8	15.0		72.3	3.9	7.3
pH	0.08	6.4	30.0	13.9	0.4	ND	73.1	3.5	5.9
		7.0		17.0	0.3		69.0	3.8	6.1
		8.0		17.0	0.3		73.2	3.6	6.1
		8.7		16.7	0.2		71.9	3.5	5.7
Temperature	0.08	7.0	27.5	15.8	6.4	ND	70.0	3.8	5.8
			30.0	15.8	0.2		68.5	3.8	5.7
			35.0	15.3	1.1		66.0	3.9	5.2
			37.5	15.5	0.2		70.5	3.7	5.8
NH ₄ Cl input concn	0.08	7.0	30.0	16.7	0.4	ND	71.2	3.8	6.4
				15.4	1.7		71.6	3.5	8.5
				10.5	22.5		69.8	3.6	6.3
				8.9	796		71.1	3.5	6.1
Glucose input concn	0.1	7.0	30.0	0.13	6.4	ND	71.0	3.5	5.6
				0.78	1.3		79.0	3.4	6.6
				5.7	1.6		77.6	3.8	5.7
				16.1	0.4		75.4	3.6	9.3
				42.4	0.1		69.4	3.5	5.3
				2.2	145		72.6	4.0	5.5
Phosphate input concn	0.14	7.0	30.0	14.3	69.5	0.01	68.9	3.9	5.5
				11.8	1.5	106	73.4	3.2	6.3
				12.9	2.6	490	73.8	3.2	6.7
				11.2	0.64	4030	67.0	3.2	8.4
Time* (h)	66	0.08	30.0	5.5	3.2	ND	72.7	3.8	7.8
	90			5.5	7.7		70.2	3.7	6.0
	136			5.7	4.3		68.5	3.8	7.0
	184			4.9	0.8		70.2	3.8	9.3
	233			4.9	0.8		67.1	4.0	6.1
	304			5.3	0.8		69.4	3.8	4.7
	352			5.1	1.1		67.1	4.0	6.4
	405			5.3	0.7		67.0	3.6	8.1

ND, Not determined.

* Time after inoculation, see Fig. 2.

Temperature. To minimize problems created by high viscosities, a dilution rate of 0.08 h⁻¹ was selected and the pH was maintained at 7. Under nitrogen limitation, growth and polysaccharide production were considerably reduced at 15 °C and 40 °C, whilst the optimum temperature for polysaccharide formation was 30 °C with a maximum glucose conversion of 59.6 % (Fig. 4). The efficiency of glucose conversion decreased markedly on either side of the optimum (45 % at 25 °C and 36.8 % at 35 °C) although the steady-state cell population remained constant over a wide temperature range (20 to 37.5 °C).

pH. The effect of pH on steady-state exopolysaccharide production was examined with nitrogen-limited cultures at 30 °C (*D* = 0.08 h⁻¹). Maximum polymer levels were obtained at pH 6.5 to 8 with a definite optimum at pH 7. Below pH 6.5 both the cell population and

Fig. 5. Effect of pH on steady-state exopolysaccharide production (Δ), glucose conversion efficiency ($\blacktriangle$), E_{520} ($\circ$), total culture protein ($\bullet$) and culture viscosity ($\blacksquare$).

polysaccharide production were reduced (Fig. 5); increasing the pH to 8.7 also resulted in a reduction in the polysaccharide level (42 %) but not in the total cell population. The conversion of glucose into exopolysaccharide reached a maximum of 51.6 % at pH 7 and fell only slightly to 48.5 % at pH 8. Under the fermentation conditions used, the pH fell to, and remained constant at, pH 6.7 in the absence of pH control. The polysaccharide varied little in overall composition regardless of environmental pH (Table 1).

Effect of medium composition on steady-state exopolysaccharide formation

The effects of nutrient levels on exopolysaccharide production in continuous culture were examined with controlled pH (7.0), temperature (30 °C) and aeration.

Nitrogen. Polysaccharide production increased with increasing nitrogen input to a maximum at 1.25 mg NH_4Cl ml^{-1} but subsequently decreased as the nitrogen levels became non-limiting (indicated by the increasing NH_4Cl content of the effluent). The steady-state cell population increased with NH_4Cl input (Fig. 6) but, as in batch culture, was decreased at concentrations in excess of 2 mg NH_4Cl ml^{-1} . The observed pattern suggested that at higher input levels factors other than the nitrogen content were growth limiting. Culture viscosity and encapsulation have been implicated in restricted nutrient transport into the cell with resultant growth limitation (Tanzer, Wood & Krichevsky, 1969; Moraine & Rogovin, 1973).

The formation of a green-yellow, extracellular, water-soluble pigment increased as the nitrogen:carbon ratio in the medium increased. The composition of the polysaccharide did not alter (Table 1).

Glucose. Increasing the glucose input concentration of the defined medium (0.5 mg NH_4Cl ml^{-1}) from 3.8 to 44.9 mg ml^{-1} had little effect on the steady-state polysaccharide production and glucose conversion efficiency of nitrogen-limited chemostat grown cells ($D = 0.1 \text{ h}^{-1}$) (Fig. 7). Under glucose limitation ($< 0.92 \text{ mg ml}^{-1}$) exopolymer could not be detected in cell-free culture supernatants by propan-2-ol precipitation and the total cell protein:total cell carbohydrate ratio was increased (2.72 and 0.95 at glucose inputs of 0.92 and 30.5 mg ml^{-1} , respectively). At higher nitrogen input levels (1.6 mg NH_4Cl ml^{-1}) increasing the glucose concentration from 1.2 to 3 mg ml^{-1} did not remove the imposed limitation. Polysaccharide was formed, with low glucose conversion efficiency due to the associated increased cell population, at glucose input concentrations above 6 mg ml^{-1} .

Fig. 6. Effect of the NH_4Cl input concentration on steady-state exopolysaccharide production (Δ), glucose conversion efficiency ($\blacktriangle$), E_{520} ($\circ$) and culture viscosity ($\blacksquare$).

Fig. 7. Effect of glucose input concentration on steady-state exopolysaccharide production (Δ), glucose conversion efficiency ($\blacktriangle$), E_{520} ($\circ$) and culture viscosity ($\blacksquare$).

Exopolysaccharide levels of 0.11 and 2.2 mg ml^{-1} were obtained with glucose inputs of 6.3 and 10.5 mg ml^{-1} respectively (glucose conversion 12.9 and 16.0 %).

Phosphate. A dilution rate of 0.14 h^{-1} was selected to minimize viscosity effects associated with the increased cell population at higher nitrogen input levels. Phosphate limitation did not increase polysaccharide production (Fig. 8). The efficiency of glucose conversion was low because of the higher initial nitrogen content of the medium and the increased dilution rate.

The polysaccharide varied little in overall composition irrespective of the pH, temperature, nitrogen, carbon or phosphate content of the growth medium (Table 1). The qualitative monosaccharide composition of complete acid hydrolysates of the exopolysaccharide samples was examined chromatographically and no significant variations were observed. In addition the acyl component of all samples analysed was constant at 9 to 12 %. The rheological characteristics of the exopolysaccharide samples were not determined and at the high shear rate used (437 s^{-1}) the apparent viscosity of the culture was related to its exopolymer content. However, variations in the solution properties of xanthan gum have been observed in preparations, formed by variant strains, which have a lower pyruvic acid content (Sandford *et al.*, 1977).

Fig. 8. Effect of phosphate input concentration on steady-state exopolysaccharide production (Δ), glucose conversion efficiency ($\blacktriangle$), E_{520} ($\circ$), total culture protein ($\bullet$) and culture viscosity ($\blacksquare$).

DISCUSSION

Continuous fermentation studies confirmed that exopolysaccharide production by *Pseudomonas* NCIB11264 was influenced by medium composition, temperature, pH, and the growth rate of the organism. Maximum steady-state levels of polysaccharide were obtained under conditions of nitrogen limitation with excess carbohydrate substrate at 30 °C and at pH 7.0. The efficiency of glucose conversion into exopolysaccharide was as high as 73 % in chemostat cultures, compared with only 30 % in batch culture.

As in batch culture, and in common with other pseudomonads (Goto, Murakawa & Kuwahara, 1973; Moraine & Rogovin, 1973), polysaccharide production was favoured by a high carbon:nitrogen ratio in the growth medium. Increasing the concentration of the carbon source when in excess did not enhance the yield of polysaccharide in either batch (Williams & Wimpenny, 1977) or continuous fermentations. The initial glucose concentration of 2.5 to 3.0 % (w/v), shown to be optimal for polysaccharide production by *Pseudomonas* NCIB11264, *Pseudomonas aeruginosa* (Goto *et al.*, 1973) and *Xanthomonas campestris* (Rogovin *et al.*, 1961) when grown in chemically defined media in batch culture, can thus be reduced to 0.5 to 1.0 % for continuous fermentations (cf. Silman & Rogovin, 1972).

The inclusion of phosphate in defined media has been shown to be necessary for maximum exopolymer formation by *Pseudomonas* NCIB11264 (Williams & Wimpenny 1977) and other *Pseudomonas* strains (Goto *et al.*, 1971, 1973). Similarly in continuous fermentations the steady-state polysaccharide levels, at a controlled optimum pH, were reduced under conditions of phosphate limitation. To obtain maximum exopolymer synthesis it was essential to maintain the pH at or near to neutrality. Slime production by *P. aeruginosa* (Goto *et al.*, 1971) and the xanthan fermentation (Moraine & Rogovin, 1973) were similarly optimal at pH 7, the rate of gum production by *Xanthomonas campestris* in continuous culture being related to culture pH (Silman & Rogovin, 1970). The fermentation efficiency may thus be increased by pH control; Moraine & Rogovin (1971) demonstrated that xanthan gum could be more efficiently produced in higher yields by continuously controlling the pH of the fermentation liquor.

The growth temperature is also critical in determining the extent of exopolysaccharide production. Although the optimum temperature for maximum cell yield of *Alcaligenes faecalis* var. *myxogenes* occurred at 32 °C, maximum polysaccharide yields were obtained at 28 °C (Harada *et al.*, 1965). Similarly the temperature optimum for the xanthan fermentation was at 28 °C and continued product formation was dependent on this tempera-

ture being maintained throughout (Moraine & Rogovin, 1973). Exopolysaccharide formation by *Pseudomonas* NCIB11264 was, however, maximum at 30 °C and the culture density was constant over the temperature range 20 to 37.5 °C. Goto *et al.* (1971) indicated that the yield of slime formed by *P. aeruginosa* was greater at 37 °C than at 25 °C whereas slime production with the strain used by Evans & Linker (1973) increased with decreasing temperature. Other bacterial strains, including certain Enterobacteriaceae (Wilkinson, Duguid & Edmunds, 1954), produce higher polysaccharide yields at lower temperatures, and mutants of *Klebsiella aerogenes* have been isolated that exhibit temperature-dependent exopolysaccharide synthesis (Norval & Sutherland, 1969).

Another factor which is believed to influence exopolysaccharide production is the availability of oxygen, although there are no quantitative data available correlating polysaccharide formation and oxygen tension. The impeller speed and gassing rate were selected so as to maintain efficient aeration in the presence of the exopolysaccharide. Lowering the aeration rate (from 500 to 400 ml min⁻¹) had no apparent effect on polysaccharide formation, although the inclusion of baffles led to increased polysaccharide yields in shaken flask cultures over a 48 h period (Williams, 1974). Wash-out from the chemostat occurred if aeration was stopped. Duguid & Wilkinson (1953) observed that anaerobiosis was specifically unfavourable to exopolysaccharide synthesis by *Aerobacter aerogenes*, whilst conditions of low aeration favoured growth and polysaccharide production by *Rhizobium meliloti* (Dudman, 1964).

During batch cultures, polysaccharide production did not commence until late in the exponential phase of growth but then continued maximally during the stationary phase when growth had ceased (Williams & Wimpenny, 1977). The generation time of the organism in defined medium during batch culture was 2.3 h and as the dilution rate approached 0.3 h⁻¹ exopolysaccharide formation decreased. The characteristics of exopolysaccharide production in batch culture were thus similar to those of a secondary metabolite. Similarly polysaccharide production by *Zoogloea ramigera* (Parsons & Dugan, 1971), *Zoogloea* MP6 (Unz & Farrah, 1976) and *P. fluorescens* (Eagon, 1956) occurred in the late exponential and stationary growth phase. During continuous culture secondary metabolites can be produced at low dilution rates where balanced growth is continuously maintained, or through nutrient imbalance. At dilution rates lower than $0.06 \times \mu_{\max}$ all or a part of the population exhibit some properties typical of the non-growing state (Pirt, 1972), and although dilution rates were thus in excess of this critical growth rate, exopolysaccharide production continued. Polysaccharide levels were highest at lower dilution rates, as were the mean residence times of the organism, but decreased with increasing dilution rate, although the cell population remained constant. At the lower dilution rates the higher polysaccharide levels may themselves be growth limiting, as Tanzer *et al.* (1969) reported that growth restriction of plaque-forming streptococci was associated with dextran formation, whilst Moraine & Rogovin (1973) proposed that increased fermentation viscosities and encapsulation were factors which contributed to growth limitation by restricting the transport of nutrients into the cell. However, in this series of experiments, fermentation conditions were pre-selected so that, in general, the polysaccharide content, and hence viscosity, of the fermentation liquor was such that efficient aeration of the culture was possible.

The practical aspects of a continuous fermentation to produce microbial exopolysaccharide on a large scale were assessed by Silman & Rogovin (1970, 1972) who were able to obtain over 80 % conversion of glucose into the exopolymer xanthan gum. Chemostat cultures of *Pseudomonas* NCIB11264 would convert over 70 % of the glucose utilized into exopolysaccharide, compared with 30 % in a 50 h batch fermentation. However, with further investigation it is possible that the efficiency of the continuous fermentation may be improved or the system modified to increase polysaccharide yields. The use of multi-stage systems for the xanthan fermentation has been suggested (Moraine & Rogovin, 1966;

Lindblom & Patton, 1967) and preliminary investigations with non-proliferating suspensions of *Pseudomonas* NCIB11264 (Williams, 1974) indicate that exopolymer formation in a two-stage system in which glucose-limited cells are transferred to a nitrogen-free polysaccharide production stage, should be considered as a means of further increasing the fermentation efficiency.

A.G.W. acknowledges the financial support of a C.A.P.S. award from the Science Research Council and Tate & Lyle Ltd.

REFERENCES

- COOPER, C. M., FERNSTROM, G. A. & MILLER, S. A. (1944). Performance of agitated gas-liquid contactors. *Industrial and Engineering Chemistry* **36**, 504-509.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for the determination of sugars and related substances. *Analytical Chemistry* **28**, 350-356.
- DUDMAN, W. F. (1964). Growth and extracellular polysaccharide production by *Rhizobium meliloti* in defined medium. *Journal of Bacteriology* **88**, 640-645.
- DUGUID, J. P. & WILKINSON, J. F. (1953). The influence of cultural conditions on polysaccharide production by *Aerobacter aerogenes*. *Journal of General Microbiology* **9**, 174-189.
- EAGON, R. G. (1956). Studies on polysaccharide formation by *Pseudomonas fluorescens*. *Canadian Journal of Microbiology* **2**, 673-676.
- EVANS, C. G. T., HERBERT, D. & TEMPEST, D. W. (1970). The continuous cultivation of microorganisms. II. Construction of a chemostat. *Methods in Microbiology* **2**, 277-328.
- EVANS, L. R. & LINKER, A. (1973). Production and characterisation of the slime of *Pseudomonas aeruginosa*. *Journal of Bacteriology* **116**, 915-924.
- FISKE, C. H. & SUBBAROW, Y. (1925). The colorimetric determination of phosphorus. *Journal of Biological Chemistry* **66**, 375-400.
- GOTO, S., ENOMOTO, S., TAKAHASHI, Y. & MOTOMATSU, R. (1971). Slime production by *Pseudomonas aeruginosa*. I. Conditions for slime production by the cellophane plate method. *Japanese Journal of Microbiology* **15**, 317-324.
- GOTO, S., MURAKAWA, T. & KUWAHARA, S. (1973). Slime production by *Pseudomonas aeruginosa*. II. A new synthetic medium and cultural conditions for slime production by *Pseudomonas aeruginosa*. *Japanese Journal of Microbiology* **17**, 45-51.
- HARADA, T., YOSHIMURA, T., HIDAKA, H. & KOREEDA, A. (1965). Production of a new acidic polysaccharide, succinoglucan, by *Alcaligenes faecalis* var. *myxogenes*. *Agricultural and Biological Chemistry* **29**, 757-762.
- LILLY, V. G., WILSON, H. A. & LEACH, J. G. (1958). Bacterial polysaccharides. II. Laboratory scale production of polysaccharide by species of *Xanthomonas*. *Applied Microbiology* **6**, 105-108.
- LINDBLOM, G. P. & PATTON, J. T. (1967). *U.S. Patent No. 3*, 328, 262.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MORAINÉ, R. A. & ROGOVIN, P. (1966). Kinetics of polysaccharide B-1459 fermentation. *Biotechnology and Bioengineering* **8**, 511-524.
- MORAINÉ, R. A. & ROGOVIN, P. (1971). Xanthan biopolymer production at increased concentration by pH control. *Biotechnology and Bioengineering* **13**, 381-391.
- MORAINÉ, R. A. & ROGOVIN, P. (1973). Kinetics of the xanthan fermentation. *Biotechnology and Bioengineering* **15**, 225-237.
- NORVAL, M. & SUTHERLAND, I. W. (1969). A group of *Klebsiella* mutants showing temperature-dependent polysaccharide synthesis. *Journal of General Microbiology* **57**, 369-377.
- PARSONS, A. B. & DUGAN, P. R. (1971). Production of extracellular polysaccharide matrix by *Zoogloea ramigera*. *Applied Microbiology* **21**, 657-661.
- PIRT, S. J. (1972). Prospects and problems in continuous flow culture of microorganisms. *Journal of Applied Chemistry and Biotechnology* **22**, 55-64.
- ROGOVIN, S. P. (1969). *U.S. Patent No. 3*, 485, 719.
- ROGOVIN, S. P., ANDERSON, R. F. & CADMUS, R. F. (1961). Production of polysaccharide with *Xanthomonas campestris*. *Biotechnology and Bioengineering* **3**, 51-63.
- SANDFORD, P. A., PITTSLEY, J. E., KNUTSON, C. A., WATSON, P. R., CADMUS, M. C. & JEANES, A. (1977). Variation in *Xanthomonas campestris* NRRL B.1459: characterisation of xanthan products of differing pyruvic acid content. In *Extracellular Microbial Polysaccharides*, A.C.S. Symposium Series, vol. 45, pp. 192-210. Edited by P. A. Sandford and A. Laskin. Washington: American Chemical Society.
- SILMAN, R. W. & ROGOVIN, S. P. (1970). Continuous fermentation to produce xanthan biopolymer: laboratory investigation. *Biotechnology and Bioengineering* **7**, 75-83.
- SILMAN, R. W. & ROGOVIN, S. P. (1972). Continuous fermentation to produce xanthan biopolymer: effect of dilution rate. *Biotechnology and Bioengineering* **14**, 23-31.
- SOLORZANO, L. (1969). Determination of ammonia in natural waters by the phenol-hypochlorite method. *Limnology and Oceanography* **14**, 799-801.
- TANZER, J. M., WOOD, W. I. & KRICHEVSKY, M. I. (1969). Linear growth kinetics of plaque-forming streptococci in the presence of sucrose. *Journal of General Microbiology* **58**, 125-133.

- UNZ, R. F. & FARRAH, S. R. (1976). Exopolymer production and flocculation by *Zoogloea* MP6. *Applied and Environmental Microbiology* **31**, 623–626.
- WILKINSON, J. F., DUGUID, J. P. & EDMUNDS, P. N. (1954). The distribution of polysaccharide production in *Aerobacter* and *Escherichia* strains and its relation to antigenic character. *Journal of General Microbiology* **11**, 59–72.
- WILLIAMS, A. R. (1969). An electromagnetic modification of the Zimm–Crothers viscometer. *Journal of Scientific Instruments* **2**, 279–281.
- WILLIAMS, A. G. (1974). *Extracellular polysaccharide production by a Gram-negative bacterial isolate*. Ph.D. thesis, University of Wales.
- WILLIAMS, A. G. & WIMPENNY, J. W. T. (1976). Exopolysaccharide production by *Pseudomonas* PBI in batch and continuous culture: effect of growth conditions. *Journal of Applied Chemistry and Biotechnology* **26**, 326–327.
- WILLIAMS, A. G. & WIMPENNY, J. W. T. (1977). Exopolysaccharide production by *Pseudomonas* NCIB11264 grown in batch culture. *Journal of General Microbiology* **102**, 13–21.
- WILLIAMS, A. G., WIMPENNY, J. W. T. & LAWSON, C. J. (1973). The production of an extracellular polysaccharide by a *Pseudomonas*-type microorganism. *Journal of General Microbiology* **77**, xiii.