FEMS Microbiology Letters 78 (1991) 285–292

Published by Elsevier
ADONIS 0378109791001423

FEMSLE 04339

Fermentation of inulin by a new strain of *Clostridium thermoautotrophicum* isolated from dahlia tubers

Wim J. Drent and Jan C. Gottschal

Department of Microbiology, University of Groningen, Haren, The Netherlands

Received 15 October 1990
Revision received 8 November 1990
Accepted 9 November 1990

Key words: Thermophilic anaerobe; Inulinase; Enrichment

1. SUMMARY

A new inulin-fermenting strain of *Clostridium thermoautotrophicum* was isolated through enrichment on dahlia tubers, and subsequent plating on agar media with undissolved inulin. Both the cell-bound and cell-free inulinase(s) functioned optimally at 60°C and at neutral pH. This new strain I1 is readily distinguished from the type strain of *C. thermoautotrophicum* with respect to fermentation products, substrate spectrum and optimum temperature for growth.

2. INTRODUCTION

Inulin occurs as a storage polysaccharide in several plant families, among which the *Graminaeae* and the *Compositae* are the most significant [1]. In the past few years much research focused on the use of this relatively cheap and abundant substrate for the microbial production of high fructose syrups [2–4]. In addition this linear polymer of fructose with one terminal glucose may be used as a substrate for various alcoholic fermentations [2,3].

With respect to thermophilic inulin degradation only cultures of aerobic bacilli have been described in the literature [5]. Therefore we have chosen this relatively abundant, biotechnologically promising polysaccharide as a model substrate to study polymer hydrolysing fermentative bacteria both in pure and in mixed culture. Especially thermostable inulinases could find application in the production of high fructose syrups because the poor solubility of inulin at low temperature is the limiting factor in obtaining high conversion ratios [6].

Using dissolved inulin as the sole source of carbon and energy a number of new, succinate forming thermophilic clostridia have been isolated recently in our laboratory. These strains exhibited relatively low activities of almost completely cell-bound inulinases (in preparation). In order to obtain other bacteria with higher inulinase-activity a number of enrichments were made using solid fragments of dahlia tubers as a source of undissolved inulin, which may stimulate selection of such organisms.

Correspondence to: W.J. Drent, Department of Microbiology, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands.

In the present paper detailed information is presented on such isolations and the properties of one of these inulin hydrolysing strains, forming distinct clearing zones on agar-plates with finely-dispersed solid inulin particles are reported.

3. MATERIALS AND METHODS

3.1. Media and cultivation conditions

Enrichments and growth studies were done in 600-ml screw-capped bottles, containing 250 ml of either a bicarbonate-buffered medium (30 mM, pH 6.8) under a N_2/CO_2 atmosphere (80/20; % v/v) or a sodium phosphate buffered medium (30 mM; pH 6.8) under a 100% N_2 atmosphere. A small amount of bicarbonate was added in this case (5 mM). The composition of the growth medium used was (g l^{-1}): NaCl, 1.2; $MgCl_2 \cdot 6H_2O$, 0.4; KCl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.15; NH_4Cl, 0.27; KH_2PO_4, 0.205; Na_2SO_4, 0.1; $NaHCO_3$, 2.52 (or NaH_2PO_4, 2.06 plus Na_2HPO_4, 2.66); $Na_2S \cdot 9H_2O$, 0.15; yeast extract, 0.025; casamino acids, 0.025; resazurin, 0.001; trace elements solution 1 ml l^{-1} (Sl 6^+ [7]) and a vitamin solution 1 ml l^{-1} [8]. The following concentrated stock solutions were sterilised separately and added aseptically to the mineral medium as required: $NaHCO_3$ (84 g l^{-1}), NaH_2PO_4 (68.6 g l^{-1}), NaH_2PO_4 (157.3 g l^{-1}), yeast extract (50 g l^{-1}), casamino acids (40 g l^{-1}) and vitamin solution. The vitamin and the trace elements solution were filter-sterilised, the other components were autoclaved (20 min, 120°C). Inulin was sterilised for 30 min at 110°C.

3.2. Enrichment and isolation

Fresh dahlia tubers with attached clay particles were cut in slices and added to a 600-ml bottle, containing 250 ml complete basal medium. After a few days of incubation at 58°C pieces of Dahlia tuber were removed from the liquid. This was done with a sterile pair of tweezers, followed by smearing the surface of these little blocs over an agar plate, containing finely dispersed, undissolved inulin particles. Some of the observed white colonies formed clearing zones, indicating the excretion of inulinase(s) hydrolysing the solid inulin particles in the agar. After repeated plating of such colonies two strains were isolated in pure culture. During the whole isolation procedure anaerobic techniques were applied, using an anaerobic chamber for plating and a 2.5-litre anaerobic jar with a N_2/CO_2 atmosphere (80/20; % v/v) for further incubations. To such jars 0.7 ml 10% Na_2S was added in order to maintain highly reduced, anaerobic conditions.

3.3. Temperature and pH optimum for growth and substrate spectrum

Screw-capped 16×125 mm Hungate tubes fitted with butyl rubber stoppers were used for growth-substrate tests and for the determination of the pH- and temperature-dependence. All growth substrates were filter-sterilised (0.2 μm) as concentrated stock solutions.

The temperature-dependence of the growth rate was measured in 10 ml bicarbonate-buffered medium with a N_2/CO_2 headspace (80/20; % v/v), whereas for the determination of the optimum pH MES-buffer was used for cultivating at low pH (4–7), and Hepes buffer was used at high pH (7–10) (100% N_2 atmosphere; in all cases a small amount of bicarbonate was added (5 mM)).

3.4. Inulinase activity

The cells were pregrown in an inulin limited chemostat run at a dilution-rate of 0.1 h^{-1} in bicarbonate-buffered medium at a pH of 6.8. Both the cell-bound and cell-free inulinase activity were measured as the appearance of reducing sugars during a 45-min incubation period of the following reaction-mixture: 0.8 ml sodium phosphate buffer (pH 6.8, 30 mM; unless stated otherwise); 0.4 ml of 6% inulin (w/v) in sodium phosphate buffer; 0.4 ml 10 mM 1,4-dithiothreitol in sodium phosphate buffer; 0.4 ml 10 mM Na_2SO_3 in sodium phosphate buffer, and 2 ml culture supernatants or suspensions of washed cells in 30 mM phosphate buffer. Sodium sulphite effectively blocked cell metabolism as no fermentation products could be detected during the enzyme assays. All the enzyme activity measurements were done under a 100% N_2 atmosphere in Hungate tubes sealed with rubber stoppers. Strictly anaerobic conditions were necessary, because incubating the

reaction-mixture under air resulted in reduced inulinase activity. The amount of reducing sugars liberated by the inulinase(s) during the enzyme assay was linear both with time and the amount of enzyme added. One unit of inulinase activity is defined as the amount of enzyme liberating 1 μmol of reducing sugars min^{-1} (U). In order to compare the inulinase activity of our isolates with other inulin-degrading microorganisms the inulinase activity is expressed as units per gram inulin degraded per liter.

3.5. *Analytical procedures*

Short-chain fatty acids, alcohols, H_2 and CO_2 were analysed gaschromatographically [9,10], whereas formate was determined colorimetrically [11].

A Shimadzu Total Organic Carbon Analyser (Model 500) was used for determination of the carbon-content, both organic and inorganic. The concentrations of fructose and glucose were measured with the method of Somogyi for reducing sugars [12]. Cells taken from a batch-culture of strain I1 in the mid-log phase were used for Gram-staining according to the method described by Gerhardt et al. [13]. Cells taken from exponentially growing cultures of *Lactococcus lactis* and *Escherichia coli* were used as controls. The Gram-character was also determined, using the KOH test of Gregersen [14]. The G + C mol% was determined by the DSM (Braunschweig, F.R.G.) through ultracentrifugation and buoyant density measurements, with the DNA of *Micrococcus luteus* as a reference.

4. RESULTS

4.1. *Isolation and characterisation*

The bacteria enriched on dahlia tubers in liquid medium formed smooth white colonies (2–3 mm), surrounded by a clearing zone when plated on agar plates with finely dispersed inulin. This indicated secretion of inulinase dissolving the inulin particles.

After several transfers pure cultures of two very similar strains were obtained. One of these (I1) was characterised in detail. The cells of this strain were rod-shaped with a width of 0.5–0.8 μm and a length of 2.0–4.0 μm (Fig. 1A). Sporulating cells could be observed in stationary phase cultures (Fig. 1a). Vegetative cells were motile by means of peritrichously inserted flagella (Fig. 1B). Thin sections of embedded cells showed the presence of a

Table 1
Summary of the major characteristics of strain I1 and the type strain of *C. thermoautotrophicum*

Characteristic	Strain I1	*Clostridium thermoautotrophicum*
Cell shape	rod	rod
Cell size (μm)	0.5–0.8 × 2.0–4.0	0.8–1.0 × 3.0–6.0
Spore shape	round	round
Spore position	terminal	terminal
Motility	+	+
Gelatin hydrolysis	weak (2 weeks)	weak (3 weeks)
Esculin hydrolysis	+	+
Cellulose degradation	−	−
Discriminating growth Substrates:		
amygdalin	+	−
cellobiose	+	−
inulin	+	−
melizitose	+	−
melibiose	+	−
raffinose	+	−
rhamnose	−	−
ribose	−	−
salicin	+	−
starch	+	−
sucrose	+	−
Fermentation products	acetate, ethanol, formate	acetate
Gas production	+(weak)	+
Thiosulfate reduction		
To sulfide	+	+
To sulfur	−	−
Nitrate reduction	+	+
Milk reaction	curd	−
Peptidoglycan type of the cell-wall	LL-DAP [a]	LL-DAP
Gram staining	+	+
KOH test	+	ND
G + C content	56.2%	53–55%
Optimum temperature for growth	63°C	55–60°C

[a] LL-Diaminopimelic acid.

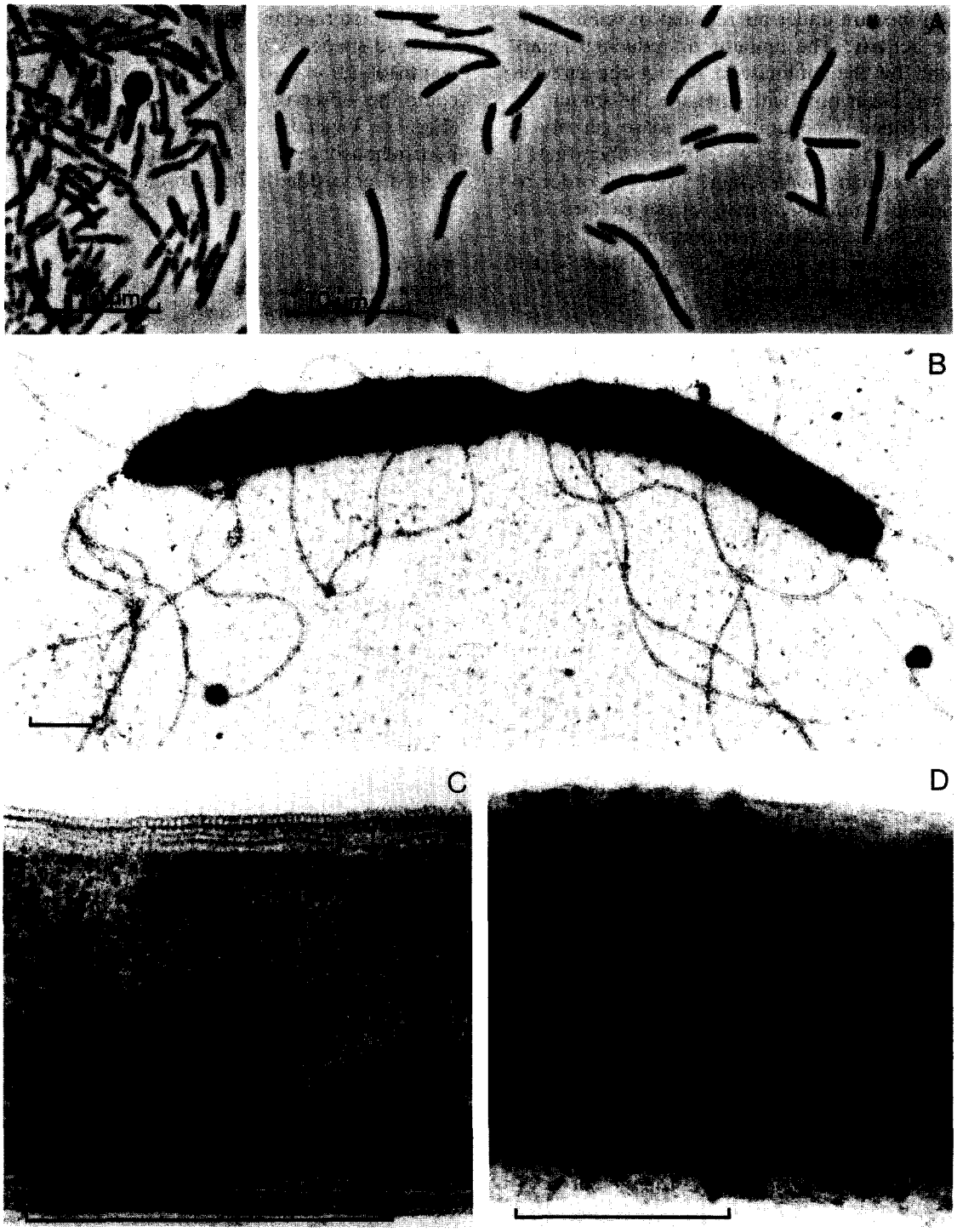

Fig. 1. (A) Phase contrast micrograph of cells of strain Il, harvested from an exponentially growing culture. (a) Phase contrast micrograph of sporulating cells, showing the presence of round terminal spores. (B) Electron micrograph of a peritrichously flagellated cell, negatively stained with uranyl acetate. (C) Regular surface layer, as revealed in an ultrathin section of an embedded cell. (D) Negatively stained fragment of the surface layer, indicating a regular arrangement of particles. Bar = 0.5 μm, unless stated otherwise.

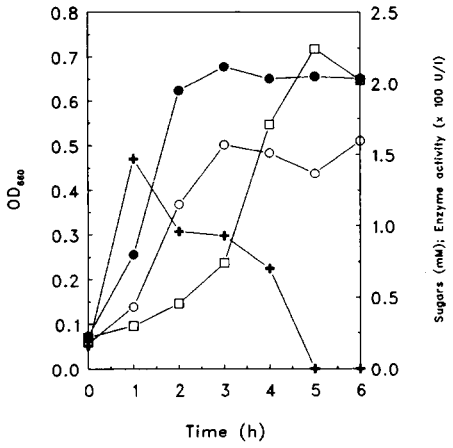

Fig. 2. Kinetics of growth and inulinase activity of strain I1 in batch-culture on inulin containing medium (1.5 g/l). (□) OD_{660}; (○), cell bound-inulinase activity; (●), cell-free inulinase activity; (+), concentration of reducing sugars in the culture fluid.

highly structured multilayered cell-surface (Fig. 1C), presumably consisting of tetragonally arranged particles (Fig. 1D).

A wide range of substrates sustained growth: amygdalin, arabinose, cellobiose, esculin, fructose, galactose, glucose, $H_2 + CO_2$, inulin, lactose, maltose, mannitol, melizitose, melibiose, methanol, raffinose, salicin, starch and sucrose. No growth was detectable with citrate, glycogen, glycerol, lactate, mannose, pyruvate, rhamnose, ribose, sorbitol, trehalose and xylose.

Additional characteristics have been summarised in Table 1.

4.2. Growth experiments

Maximum exponential growth of strain I1 in batch-culture on inulin was preceded by a substantial formation of inulinase, resulting in accumulation of reducing sugars during the early logarithmic phase (Fig. 2). This suggested that the inulin-hydrolysing capacity is greater than strictly needed for growth. A μ_{max} of 0.8 ± 0.1 h^{-1} was calculated from batch culture growth curves.

The curves describing the relationship between temperature and growth-rate on fructose and inulin almost coincided. Only a small difference in the optimum temperature for inulin and for the monomer fructose was observed (58 and 62 °C, respectively; Fig. 3A). Growth on inulin and fructose was possible over a wide pH range (4–10), with an optimum around neutral for both substrates (Fig. 3B). The μ_{max} determined in MES- or Hepes-buffered medium at pH 7 was somewhat lower than in bicarbonate buffered medium (0.4 h^{-1} and 0.5 h^{-1}, respectively).

The fermentation balance in the exponential growth-phase was (mmol): 1 fructose → 0.42 for-

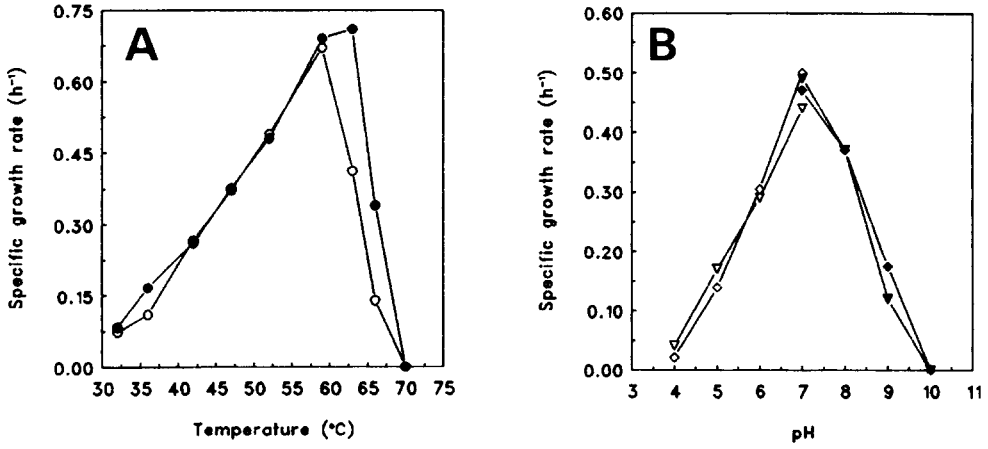

Fig. 3. Effect of temperature and pH on the specific growth rate of strain I1. (A) Effect of the temperature on the specific growth rate, using inulin (1.5 g/l) or fructose (10 mM) as the carbon- and energy source. (○), μ_{max} on inulin; (●) μ_{max} on fructose. (B) Effect of pH on the specific growth rate of strain I1, using inulin (1.5 g/l) or fructose (10 mM) as the carbon- and energy source. (▽), μ_{max} on inulin using MES buffer; (▼), μ_{max} on inulin using Hepes buffer; (◇), μ_{max} on fructose using MES buffer; (◆), μ_{max} on fructose using Hepes buffer.

mate + 0.68 acetate + 1.27 ethanol + 1.0 H_2 + 0.97 CO_2 + 0.60 cell material (cell carbon). Inulin and glucose gave very similiar results (data not shown).

4.3. Some characteristics of the inulin hydrolysing enzyme-system

In an inulin-limited continuous culture, run at a dilution rate of 0.1 h^{-1}, only 15% of the total inulinase activity was detected in the surrounding liquid. Both the cell-bound and cell-free inulinase were shown to have optimal activity between 58 and 65°C and at pH values between 6.5 and 7.2 (data not shown). At a temperature of 60°C no loss of enzyme activity was observed over a period of at least 3 h. At 65°C the activity dropped gradually to approximately 50% after 1 h and below 5% after 3 h of incubation.

5. DISCUSSION

Strain I1 belongs to the species *C. thermoautotrophicum* on the basis of the following characteristics [15]: it exhibits a similar cell morphology as the type strain, it possesses a cell wall peptidoglycan type A3v (LL-diaminopimelic acid with a glycine interpeptide bridge), it is capable of growth with hydrogen plus carbon dioxide as the sole carbon and energy source, does not produce butyrate or lactate, produces round spores resulting in a terminal swelling of the cell, grows with methanol, hydrolyses gelatin weakly, utilizes a wide range of carbohydrates and it has a G + C content of 56.2% (Table 1). A number of differences with respect to the spectrum of fermentable carbohydrates, fermentation products and optimum temperature for growth distinguish strain I1 from the type strain of *C. thermoautotrophicum* (Table 1).

The use of a high temperature and a solid substrate for enrichment and plating resulted in the isolation of an inulin degrading strain with potentially biotechnologically interesting characteristics, especially with respect to optimum temperature, thermostability and the extent of inulinase excretion. The thermostability and temperature optimum of the inulinases produced by strain I1 belong to the highest described in the literature so far [2]. The enzymes of strain I1 are more thermostable than the biotechnologically important yeast *Kluyveromyces marxianus* [4], and comparable to the inulinases, which have been obtained from *Aspergillus niger* [6]. At 60°C the inulinase activity of *K. marxianus* was reduced by 50% after 30 min of incubation, whereas no loss of inulinase-activity of strain I1 and *Aspergillus niger* could be detected after three h of incubation. However, the yeast *K. marxianus* exhibits superior properties with respect to the quantity of inulinase produced, especially when the yeast is cultivated in continuous culture at low dilution rates (*C. thermoautotrophicum*: 207 U g inulin degraded^{-1} l^{-1}; *K. marxianus* in batch culture: 21200 U g inulin degraded^{-1} l^{-1} [16]; *K. marxianus* in chemostat: 28080 U g inulin degraded^{-1} l^{-1} [4]). The inulinase activity of *C. thermoautotrophicum* in batch-culture is comparable with that of thermophilic bacilli (approximately 140 U g inulin degraded^{-1} l^{-1} [5]). It is much higher than found for thermophilic succinate forming clostridia enriched on dissolved inulin and mesophilic bacteria like *C. acetobutylicum* (6.9 U g inulin degraded^{-1} l^{-1} (in preparation) and 21.8 U g inulin degraded^{-1} l^{-1} [17], respectively).

ACKNOWLEDGEMENTS

We are grateful to K. Sjollema and J. Zagers for the preparation of the electron micrographs. The investigations were supported by the Program Committee for Industrial Biotechnology.

REFERENCES

[1] Beck, R.H.F. and Praznik, W. (1986) Starch 38, 391–394.
[2] Vandamme, E.J. and Derycke, D.G. (1983) Adv. Appl. Microbiol. 29, 139–176.
[3] Fuchs, A., de Bruyn, J.M. and Niedeveld, C.J. (1985) Antonie van Leeuwenhoek 51, 333–351.
[4] Rouwenhorst, R.J., Visser, L.E., Van der Baan, A.A., Scheffers, W.A. and Van Dijken, J.P. (1988) Appl. Environ. Microbiol. 54, 1131–1137.
[5] Allais, J.J., Hoyos-Lopez, G., Kammoun, S. and Baratti, J.C. (1987) Appl. Environ. Microbiol. 53, 942–945.
[6] Uhm, T. and Byun, S. (1987) Biotechnol. Lett. 9, 287–290.
[7] Pfennig, N. and Lippert, K.L. (1966) Arch. Microbiol. 55, 245–256.

[8] Heijthuijsen, J.H.F.G. and Hansen, T.A. (1986) FEMS Microbiol. Ecol. 38, 57–64.
[9] Nanninga, H.J. and Gottschal, J.C. (1985) FEMS Microbiol. Ecol. 31, 261–269.
[10] Laanbroek, H.J., Geerligs, H.J., Sijtsma, L. and Veldkamp, H. (1984) Appl. Environ. Microbiol. 47, 329–334.
[11] Lang, E. and Lang, H. (1972) Z. Anal.Chem. 260, 8–10.
[12] Somogyi, M. (1952) J. Biol. Chem. 195, 19–23.
[13] Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R. and Philips, G.B. (1981) Manual of Methods for General Bacteriology, American Society of Microbiology, Washington DC.
[14] Gregersen, T. (1978) Eur. J. Appl. Microbiol. Biotechnol. 5, 123–127.
[15] Cato, E.P., George, W.L. and Finegold, S.M. (1986) in Bergey's Manual of Systematic Bacteriology Vol. 2 (Sneath, P.N.A., Mair, N.S., Sharpe, M.E. and Holt, J.G., Eds.), pp. 1141–1200, Williams & Wilkins Co., Baltimore, MD.
[16] Parekh, H.A. and Margaritis, M. (1985) Appl. Microbiol. Biotechnol. 22, 446–448.
[17] Looten, P., Blanchet, D. and Vandecasteele, J.P. (1987) Appl. Microbiol. Biotechnol. 25, 419–425.