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Effect of culture conditions on growth and esterase production by the moderate thermophile *Bacillus circulans* MAS2

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Growth and esterase production (activity on *p*-nitrophenyl caprylate) by the newly isolated *Bacillus circulans* MAS2 bacterial strain were studied. The growth rate at 50°C was high (0.9 h^{-1}) on LB medium with glucose added. Esterase production followed growth with the majority of activity being intracellular during exponential growth phase. During stationary phase, the esterase activity was released in the culture medium. The strain was able to grow at 35– 55°C with maximum growth rate at 50°C, showing a pattern typical of a moderate thermophile. Growth occurred at pH 6–9 with a maximum at 8, with a similar pattern for the esterase production. Addition of glucose, fructose, sucrose or sodium acetate greatly promoted both growth and esterase production while starch, inulin, tributyrin or glycerol showed no effect. Complex nitrogen sources such as tryptone or yeast extract increased growth and esterase production while mineral sources (ammonium chloride or sulfate), glycine or glutamate showed no effect. An increase of tryptone plus yeast extract and glucose concentrations stimulated growth and esterase production which reached 160 U L^{-1}.

Keywords: esterase; *Bacillus circulans*; bacterial growth; enzyme production

Introduction

Esterases (EC 3.1.1.1) and lipases (EC 3.1.1.3) are enzymes with a great potential for biotechnological applications such as ester synthesis as flavors for the food industry [11], modification of the physico-chemical properties of triglycerides in the fat and oil industry [15] and preparation of optically pure bioactive molecules for the pharmaceutical industry [3,14]. On a practical point of view, enzymatic processes which can be run at high temperatures are attractive for several reasons: (a) the reaction rates are increased; (b) the medium viscosity is lower; (c) the substrate solubility is increased; and (d) the microbial contamination probability is lower [2]. For these reasons, thermostable enzymes are quite useful and there is a continuous search for new enzymes with the required technological properties.

In this context, several esterases have attracted attention such as pig liver esterase, which is known for its broad substrate specificity [7,16,17] and the carboxylesterase from *Bacillus coagulans* [14]. However, there are few industrial applications of esterases, compared for instance to lipases, mainly because of the lack of availability of these biocatalysts, in sufficient amounts, for organic chemists [5].

Usually the search for thermostable enzymes is made by using thermophilic or hyperthermophilic microorganisms. In our laboratory, we have tested moderate thermophiles as an alternative source for new thermostable esterases or lipases. Thirty-nine newly isolated strains growing at 50°C on a mineral medium containing triolein as sole carbon source, produced lipolytic enzymes, as tested by *p*-nitrophenyl caprylate (pNPC8) hydrolysis [8]. Among these, strain MAS2 identified as a *Bacillus circulans*, showed the highest extracellular esterase activity. This activity was quite thermostable (100% recovery after 1 h at 70°C) and active at alkaline pH which makes it attractive for applications [9].

In this work, we report the effect of temperature, pH, carbon and nitrogen sources on both growth and esterase production.

Materials and methods

Organism and culture conditions

Strain MAS2 was isolated in our laboratory [8] from a soil sample using an enrichment culture at 50°C on a mineral medium containing triolein as sole carbon source. The strain was grown on LB medium until exponential phase and stored at −80°C in 20% (v/v) glycerol. Growth and esterase production were studied on LBG medium (LB with glucose) with the following composition: 10 g L^{-1} tryptone (Difco, Detroit, MI, USA), 5 g L^{-1} yeast extract (Organotechnie, La Courneuve, France), 10 g L^{-1} NaCl (LB medium, [12]) and 10 g L^{-1} glucose. Glucose was sterilized separately at 110°C for 15 min and LB was sterilized at 120°C for 15 min. The pH of the medium was adjusted to 7.5 by the addition of NaOH.

For the effect of carbon source, the tryptone and yeast extract were considered as supplying only nitrogen and glucose was replaced by other carbon sources as indicated in the text. The substitution was made on the basis of a constant mole of carbon per liter basis, ie 0.33 mol L^{-1}. The following additions were made (per liter): fructose 9.9 g, sucrose 9.4 g, starch 9 g, inulin 9 g, tributyrin 6.7 g, triolein

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5.1 g, Tween 80 6.8 g, glycerol 10.1 g and sodium acetate 22.5 g. The pH was adjusted to 8.1 with NaOH.

For the effect of nitrogen source, tryptone and yeast extract of LBG were replaced by other nitrogen sources at the same molar nitrogen concentration of 0.126 mole of N per L by addition of (per liter): yeast extract 19.2 g, tryptone 13.4 g, peptone 10.9 g, ammonium acetate 9.7 g, ammonium chloride 6.7 g, ammonium sulfate 8.3 g, glutamate 18.5 g and glycine 9.5 g. For yeast extract, tryptone and peptone the substrate concentration S (in g L^{-1}) was calculated using the formula $S = 0.126$ x $(14/N)$ where N is the total nitrogen content (in %, w/w) given by the supplier (yeast extract 9.2%, tryptone 13.1% and peptone 16.2%). The pH was adjusted to 8.1 with NaOH.

All the cultures were run in triplicate in 250-ml Erlenmeyers containing 25 ml of liquid medium, incubated at 50°C and agitated at 110 strokes per min.

Enzyme assay

Esterase activity was assayed by measuring the rate of hydrolysis of *p*-nitrophenyl caprylate (pNPC8) (Sigma Chemicals, St Louis, MO, USA) at 37°C according to Kordel *et al* [10]. One volume of 16.5 mM pNPC8 in 2-propanol was mixed with 9 volumes of 50 mM Tris-HCl pH 8.0, containing 0.4% (w/v) Triton X-100 (Fluka/Sigma Aldrich, Saint Quentin, Fallavia, France) and 0.1% (w/v) arabic gum (Sigma). To 1.35 ml of this mixture equilibrated at 37°C in a 3-ml cuvette of the spectrophotometer (Shimadzu UV-160A, Roucaire, Courtaboeuf, France), 0.15 ml of the enzyme solution was added. The absorbance at 410 nm was read against a blank without enzyme and monitored continuously. The concentration of liberated *p*-nitrophenol (pNP) was calculated using an extinction coefficient of 12.75 10^6 cm^2 mol^{-1}. This value was determined using standard solutions of pNP. For each assay, three different enzyme quantities were tested and activity was calculated from the slope of the curve of variation of absorbance per min against enzyme amount. One enzyme unit was the amount of enzyme liberating 1 μmol of pNP per min under the conditions used. Activity was assayed in both culture medium and centrifuged cells. In this latter case the cells were collected (5000 × *g*, 4°C, 10 min) and washed twice with 20 mM Tris-HCl buffer pH 8.0.

Analytical methods

Growth was followed by the absorbance at 660 nm. At 24 h, one absorbance unit was equivalent to a biomass of 2.1 g L^{-1} of culture medium. No changes in cell morphology were observed under the conditions used. Therefore, absorbance was used for both the measures of cell growth and cell yield. Glucose was assayed enzymatically using the reagent: peroxidase (20 μg ml^{-1}) (Sigma), glucose oxidase (180 μg ml^{-1}) (Sigma), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (1 mg ml^{-1}) in 0.1 M sodium phosphate buffer pH 7.0. The sample (0.5 ml) was mixed with reagent (3 ml) and the mixture was incubated at room temperature for 40 min in the dark. Absorbance was read at 610 nm against a blank.

Results

Growth and esterase production

B. circulans MAS2 was cultivated at 50°C on LBG medium and growth was followed by absorbance at 660 nm. Growth was fast (specific growth rate of 0.9 h^{-1}) on this medium with a maximum of biomass reached for 12 h and a slow decrease afterward due to cell lysis (Figure 1a). Surprisingly, glucose was not utilized during the first 5 h, but its concentration decreased slowly from 10 to 15 h. Only 20% of the glucose was consumed by the strain at the end of fermentation, suggesting that glucose was not the limiting substrate in LBG. The intracellular esterase activity increased in parallel to growth until 10 h and it decreased for longer incubation times. This decrease was parallel to an increase in extracellular activity suggesting that lysis released the cell protein into the medium. This hypothesis was further confirmed by the fact that the sum of extra- and intra-cellular activity was almost constant (Figure 1b). The esterase specific activity (in esterase units per absorbance units) increased during the exponential growth phase and remained constant later on, suggesting the occurrence of some regulation of esterase production. For further experiments, the cultures were carried out for 18 h to get the maximum activity as extracellular. On a practical point of view, enzyme recovery is much easier from culture medium than from whole cells which validates this approach.

Effect of temperature

B. circulans MAS2 was grown at temperatures from 30 to 60°C on LBG medium. The variation of the specific growth rate is shown in Figure 2. No growth was observed at 30°C indicating that the strain is a thermophile rather than a thermotolerant bacterium. The maximum growth rate (1.1 h^{-1}) was at 50°C and no growth was detected at 60°C which classified the strain as a moderate thermophile. The absorbance at 660 nm and esterase activity after 18 h of culture are shown in Table 1. The final biomass concentration was slightly affected by temperature; it was maximum at 50°C. All the observed values were much higher than those measured after 5 h, suggesting that cell lysis was low under these conditions. The total esterase activity and the biomass were also maximum at 50°C, suggesting a direct correlation between growth and enzyme production. This result is also suggested by the small variation of specific activity (esterase units per absorbance unit) which varied from 11 to 30. The amount of esterase activity in the culture medium varied with temperature from 10 to 40% with a maximum at 45–50°C.

Effect of pH

B. circulans MAS2 was grown at pH 5.6–9.1 at 50°C on LBG medium. The variation of the specific growth rate is shown in Figure 3. No growth was observed at pH 5.6 or 9.1 and the specific growth rate was maximum (1.2 h^{-1}) at pH 8. Thus, strain MAS2 is slightly alkalophilic. The absorbance at 660 nm and esterase activity after 18 h of culture are shown in Table 2. The final biomass concentration increased with pH and was maximum at pH 8.9. The total activity followed a similar variation while the specific

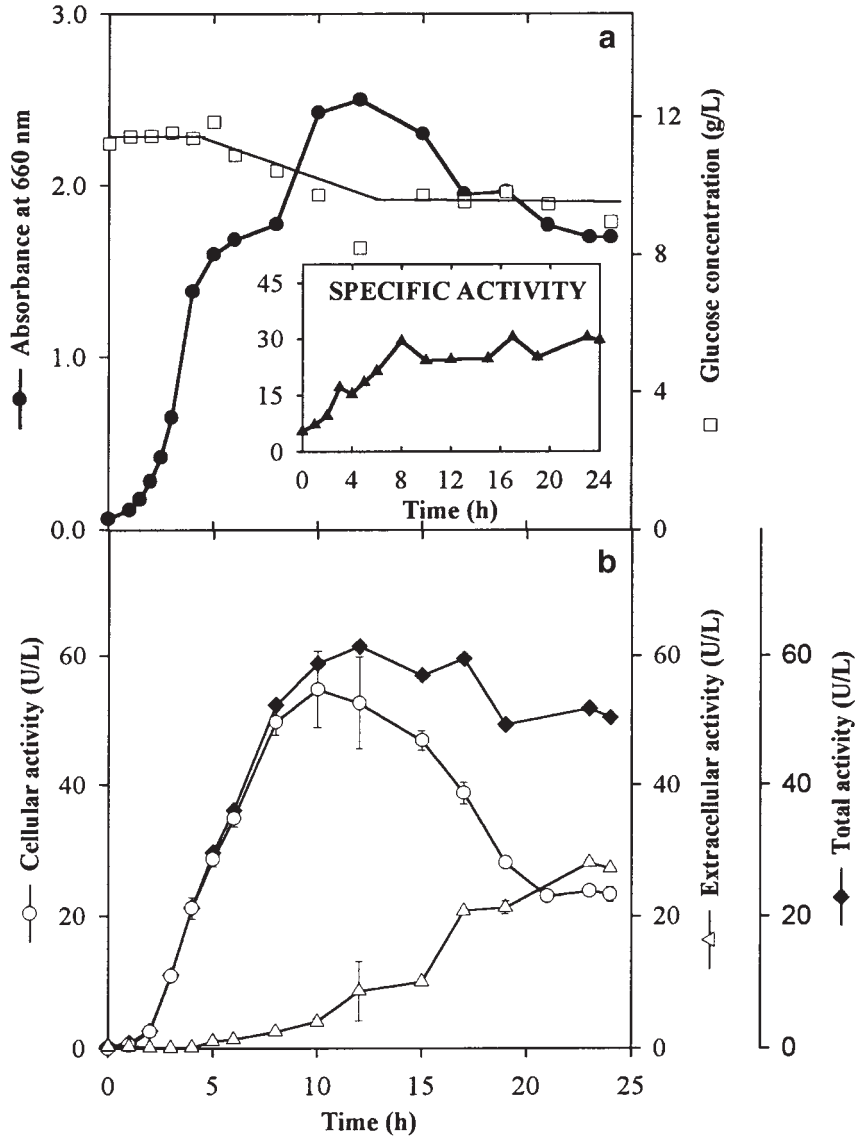

Figure 1 Growth and esterase production by *B. circulans* MAS2. (a) Growth kinetics and glucose concentration. (b) Total, cellular and extracellular esterase activities. Insert: specific activities expressed in U L^{-1} OD. Values are averages of results from three independent cultures.

activity was almost constant. The percentage of activity in culture medium increased with pH with the exception of pH 6.1. In this case, a significant cell lysis was observed which may increase the amount of extracellular esterase activity.

Influence of carbon source

In a previous work [9], we showed that glucose addition to LB medium greatly stimulated both cell growth and esterase production while neither Tween 80 nor triolein stimulated esterase production. In LBG medium, glucose was replaced by another carbon source at the same concentration in moles of C per L (see Materials and Methods for details). The medium pH was increased to 8.1, the optimum for growth (Figure 3). The absorbance at 660 nm and esterase activity after 18 h at 50°C are shown in Table 3. The strain grew and produced esterase on each medium tested. The best results (final biomass and esterase activity) were observed with fructose, sucrose or acetate additions to LB. In contrast, starch, tributyrin or glycerol additions showed no effect. The activity was mainly (60–83%) intracellular except for glycerol (14%).

Effect of nitrogen source

The effect of nitrogen source was tested on LBG by removing the yeast extract and tryptone and replacing them with selected nitrogen sources. The nitrogen concentration was maintained at 0.126 mol of N per L. The absorbance at 660 nm and esterase activity after 18 h at 50°C are shown in Table 4. Growth and esterase production were greatly affected by the nature of the nitrogen source. None of the mineral or organic sources tested were efficient for growth and esterase production with the exception of rich sources such as peptone, tryptone and yeast extract. The best results were observed for yeast extract and tryptone addition, ie with LBG medium. The esterase activity was mainly intracellu-

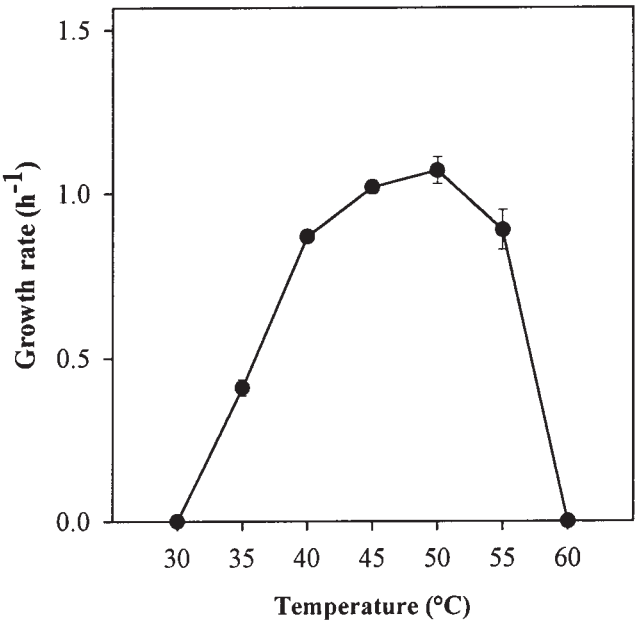

Figure 2 Effect of temperature on specific growth rate of *B. circulans* MAS2 grown on LBG medium at pH 7.5. Values are means of three different cultures.

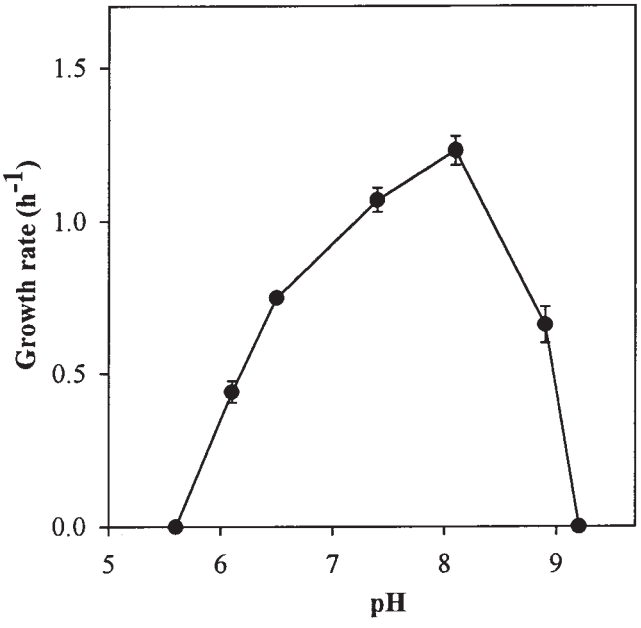

Figure 3 Effect of medium pH on specific growth rate of *B. circulans* MAS2 grown on LBG medium at 50°C. Values are means of three different cultures.

Table 1 Effect of temperature on growth and esterase production of *Bacillus circulans* MAS2

Temperature (°C)	Absorbance at 660 nm (absorbance units)	Total activity (U L^{-1})	Specific activity (U L^{-1} OD)	Activity in supernatant (%)
35	1.0	19.2 ± 0.7	19	12
40	0.7	8.0 ± 1.7	11	17
45	0.8	24.2 ± 2.8	30	40
50	1.7	42.2 ± 1.5	25	30
55	1.4	32.0 ± 3.5	23	25

Cells were grown on LBG medium for 18 h. Activity was assayed in the culture supernatant and cellular fraction. Values are means of three different cultures.

Table 2 Effect of pH on growth and esterase production of *Bacillus circulans* MAS2

pH	Absorbance at 660 nm (absorbance units)	Total activity (U L^{-1})	Specific activity (U L^{-1} OD)	Activity in supernatant (%)
6.1	0.8	18.6 ± 1.1	23	55
6.5	1.4	42.0 ± 1.6	30	24
7.4	1.7	42.5 ± 1.5	25	29
8.1	2.3	69.0 ± 4.6	30	44
8.9	3.1	68.0 ± 2.2	22	59

The organism was grown on LBG medium for 18 h at 50°C. Activity was assayed in the culture supernatant and cellular fraction. Values are means of three cultures.

lar with rich nitrogen sources and extracellular for mineral source or glycine.

Effect of medium concentration

The concentrations of the components of LBG medium were varied in order to determine the optimal ratio of carbon and nitrogen sources: three tryptone plus yeast extract concentrations were used (15, 30 and 60 g L^{-1}) with three glucose concentrations (15, 30 and 40 g L^{-1}). The composition of the media tested (M2–M7), final absorbance (18 h at 50°C) and esterase activity are shown in Figure 4. Medium M1 corresponds to LBG. Increasing the glucose concentration up to 40 g L^{-1} (media M2 and M3) did not increase growth and esterase production, which confirms

Table 3 Effect of carbon source on growth and esterase production of *Bacillus circulans* MAS2

Carbon sources	Absorbance at 660 nm (absorbance units)	Total activity (U L^{-1})	Specific activity (U L^{-1} OD)	Activity in supernatant (%)
LB	0.5	24.6 ± 1.5	55	19
LBG[a]	2.0	55.1	28	42
Fructose	2.6	73.0 ± 2.6	28	24
Sucrose	1.8	63.5 ± 2.5	35	40
Starch	0.4	12.6 ± 1.4	33	31
Inulin	1.0	29.5 ± 1.0	30	17
Tributyrin	0.7	11.8 ± 2.5	16	21
Glycerol	0.4	9.4 ± 0.4	26	86
Sodium acetate	1.2	57.2 ± 2.3	47	20

[a] Data from Figure 1. *B. subtilis* MAS2 was grown at 50°C for 18 h on LB medium with the addition of the indicated carbon sources at a concentration of 0.33 moles of carbon L^{-1}. Activity was assayed in the culture supernatant and cellular fraction. Values are means of three cultures.

Table 4 Effect of nitrogen source on growth and esterase production of *Bacillus circulans* MAS2

Nitrogen sources	Absorbance at 660 nm (absorbance units)	Total activity (U L^{-1})	Specific activity (U L^{-1} OD)	Activity in supernatant (%)
LBG	2.1	68.1 ± 6.9	32	48
Yeast extract	2.8	86.0 ± 13.3	31	35
Tryptone	1.1	55.6 ± 5.7	51	29
Peptone	0.7	16.1 ± 2.0	24	28
Ammonium acetate	0.03	4.4 ± 0.4	nc[a]	84
Ammonium sulfate	0.05	4.6 ± 0.8	nc	100
Ammonium chloride	0.07	7.3 ± 0.9	nc	49
Glutamate	0.3	9.3 ± 1.0	27	47
Glycine	0.02	9.2 ± 0.8	nc	97

[a]nc = not calculated, low absorbance values.
Cells were grown on basal medium (containing glucose 10 g L^{-1} and NaCl 10 g L^{-1}) with the addition of the indicated nitrogen source at a concentration of 0.126 moles of nitrogen L^{-1}. Activity was assayed in the culture supernatant and cellular fraction after 18 h at 50°C. Values are means of three cultures.

the conclusion that glucose is not a limiting substrate in these conditions. By contrast, both growth and esterase production were increased when the tryptone plus yeast extract concentration was increased (media M4, M5, M6 and M7) independently of the C/N ratio. The amount of intracellular activity increased from M1 to M7. Esterase production was increased 2.5 times from LBG (M1) to M7.

Discussion

B. circulans MAS2 grew fast on LBG medium with a doubling time of 46 min. The glucose of LBG medium was not completely utilized during growth. A similar situation has been reported for some strains of the thermophile *Thermus* [1]. Therefore, although glucose was not the substrate limiting growth, its addition was necessary for efficient enzyme production [9]. Esterase biosynthesis during growth seemed to be regulated since the specific activity increased during the exponential growth phase. However, the nature of this regulation is not known. Most of the esterase activity was intracellular until it reached maximum value at 12 h. After this time, activity in the culture medium began to increase significantly with a parallel decrease of the intracellular activity. Since the total esterase activity (extra plus intracellular) remained constant, the most probable explanation is that cells in stationary phase released the enzyme by either a specific mechanism or, more likely, by cell lysis. Evidence for lysis is provided by the diminution of absorbance. It is preferable to have the enzyme in the supernatant rather than in cells since enzyme recovery is much easier from culture medium than from whole cells.

The strain was able to grow at 35–55°C with maximum growth at 50°C. This pattern is typical of moderate thermophiles. Other strains isolated in similar conditions in our laboratory also belong to the same microbiological group [6]. Other *B. circulans* strains showed temperature patterns slightly lower: the strain isolated by Elwan *et al* [4] grew from 25 to 45°C while that isolated by Sztajer and Maliszewska [18] showed maximum growth at 42°C. The production of esterase activity varied with temperature similarly to growth, with a maximum activity at 50°C. *B. circulans* MAS2 grew at pH 6–9 with an optimum growth rate at pH 8, similar to that reported for other *B. circulans* strains [4,18].

The addition of glucose, fructose, sucrose or acetate to basal LB medium greatly stimulated growth and esterase production, while starch, inulin, tributyrin and glycerol had no effect. This result suggests the lack of catabolite repression by the sugars on esterase synthesis, contrary to what was observed with *Bacillus* strains LCB38, LCB39 and LCB40 by Fakhreddine *et al* [6], after glucose addition in a mineral medium containing Tween 80 as carbon

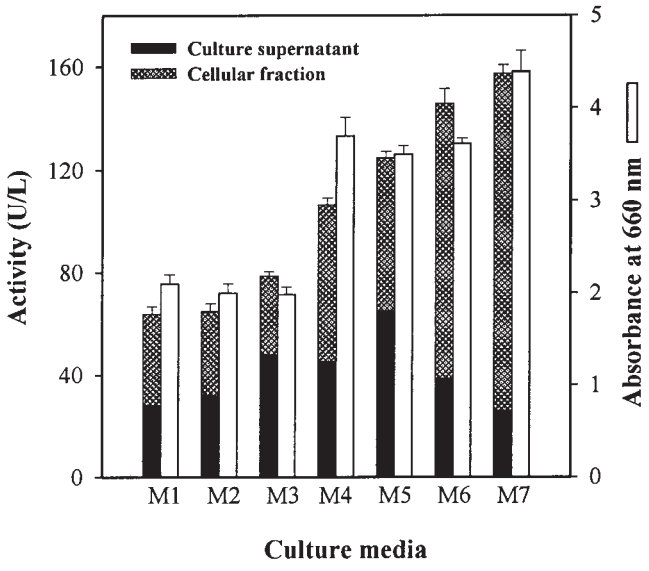

Figure 4 Effect of tryptone, yeast extract and glucose concentrations on growth and esterase production of *B. circulans* MAS2. M1 was LBG medium. In M2 and M3 the glucose concentration was increased to 20 and 40 g L^{-1}. In M4 and M5, the tryptone and yeast extract concentrations were 20 and 10 g L^{-1}, respectively, in both media while the glucose concentration was 10 and 20 g L^{-1} respectively. For M6 and M7, the tryptone and yeast extract concentrations were 40 and 20 g L^{-1}, respectively, in both media while the glucose concentration was 10 and 40 g L^{-1} respectively. The cells were grown at 50°C and pH 8.1 for 18 h. Values are means of three different cultures.

source. Contrary to our results, esterase production by *B. circulans* Bac1 was higher on starch than on glucose suggesting different regulations in these two strains [18]. The activity on fructose and sucrose (73 and 64 U L^{-1}, respectively) is similar to that reported for *B. subtilis* NRRL 365 on the same substrates [13]. When tributyrin was added to LB, growth was not affected but esterase production decreased twice. This result agreed with the lack of induction of esterase synthesis by this lipolytic substrate, confirming our previous report of lack of effect of Tween 80 or Triolein additions [9] and similar to the results of Elwan *et al* [4] for *B. circulans.*

Mineral nitrogen sources did not promote cell growth as well as glycine and, to a lesser extent, glutamate. This result suggests that the strain is auxotroph for at least one component of the rich medium. Therefore, changing the nitrogen source to a mineral one excluded this essential component and no growth occurred. These conclusions are supported by the positive effect of increasing the concentrations of tryptone and yeast extract in the culture medium, which favored growth as expected if one nutrient in LBG was present at a limiting concentration.

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