Journal of Industrial Microbiology, 12 (1993) 129–135
© 1993 Society for Industrial Microbiology 0169-4146/93/$09.00
Published by The Macmillan Press Ltd

Protease and lipase production by a strain of *Serratia marcescens* (532 S)

C. Henriette, S. Zinebi, M. F. Aumaitre, E. Petitdemange, H. Petitdemange

Laboratoire de Chimie Biologique 1, Université de Nancy 1, France

(Received 26 February 1993; revision received 22 May 1993; accepted 5 June 1993)

Key words: Protease; Lipase; Hydrolase; Catabolic repression

SUMMARY

Production of both exolipase and exoprotease activities by *Serratia marcescens* 532 S isolated from an aerobic fixed-biomass reactor were strongly influenced by nutritional factors which acted as inducers or repressors. In batch culture, protease and lipase activities were produced after the exponential phase. NH_4Cl, amino acids and simple carbon sources caused repression of protease activity. At a concentration of 1.5 g L^{-1}, the individual addition of maltose, mannitol, acetate, fructose or glucose, repressed exoprotease production, with the greatest effect by glucose. An inverse relationship existed between exoprotease synthesis and increasing glucose concentrations. Lipids activated lipase production, the most significant increase occurred when Tween 80 was added in the medium. Thus, glucidolytic, proteolytic and lipolytic activities could be efficiently expressed in batch cultures only successively.

At low dilution rate of chemostat cultures with a constant glucose input concentration of 2 g L^{-1}, glucidolytic, proteolytic and lipolytic activities were produced, but did not have the same regulation: at D values < 0.08 h^{-1}, the level of protease activity dropped while that of lipase showed a corresponding increase. Above these values, increasing D led to a decrease of the two hydrolase activities, at the level of the specific activities as well as in the specific rate of biosynthesis of each enzyme. Similar results were obtained in chemostat culture with a constant specific growth rate of 0.04 h^{-1} with increasing glucose input concentrations, i.e. protease and lipase activities decreased when the specific glucose uptake rates were enhanced.

INTRODUCTION

Microbial sources of protease and lipase occurring simultaneously are of great importance for industrial applications, e.g. detergents, dairy food processing and water treatment. A characteristic trait of many strains of *Serratia marcescens* is that they produce extracellular enzymes, including nuclease, protease [6, 1], chitinase and lipase [5, 7]. We have isolated *Serratia marcescens* 532 S from an aerobic fixed-biomass reactor [8], which when grown on agar plates containing either carbohydrates, proteins or lipids, expressed respectively, glucidolytic, proteolytic and lipolytic activities. Our interest was focused on the optimization of the hydrolytic capability of this bacterial strain in a mixed medium. It has been reported that the production of extracellular hydrolase by strains of gram-negative bacteria can be strongly influenced by culture conditions [2, 13, 15], but no information is available concerning the behavior of such bacteria exposed simultaneously to glucose, and to protease and lipase substrates. The aim of this study is to characterize proteolytic and lipolytic activity and regulation, and thereby learn whether these two activities can be expressed efficiently at the same time during glucose utilization, first in batch cultures and then in a chemostat at different specific growth rates or with different glucose inputs.

Correspondence to: H. Petitdemange, Laboratoire de Chimie Biologique 1, Université de Nancy 1, B. P. 239, 54506 Vandoeuvre-les-Nancy, Cedex, France.

MATERIALS AND METHODS

Microorganisms

The microorganism used was one of a large number of isolates obtained from an aerobic fixed-bed reactor [8]. It was identified as a non-pigmented *Serratia marcescens*. The classification was based on gram stain, catalase and oxidase reaction, and the use of API 20 E (Api System S.A., Montalieu-Vercieu, France). The organism, named *Serratia marcescens* 532 S, was maintained on R_2A agar (Difco, Detroit, USA) slants and stored at 4 °C.

Growth media

The growth medium, R_2A, consisted of a basal salt solution containing (g L^{-1}) $MgSO_4 \cdot 7\ H_2O$, 0.05; K_2HPO_4, 0.3; supplemented with proteose peptone, 0.5; casamino acids, 0.5; starch, 0.5; pyruvate, 0.5; glucose, 0.5 and yeast extract, 0.5. R_2A limited medium was composed of the basal salt solution described previously, containing 0.5 g L^{-1} of yeast extract, and only one nitrogen and one carbon source, which were added respectively in the form of casein (Hammarsten-Serva, Heidelberg, Germany), casamino acids (Difco), yeast extract (Difco), NH_4Cl, and glucose, glycerol, maltose, mannitol, acetate, fructose, Tween 80, oleate, palmitate, stearate, tributyrin, sunflower oil, glycogen and laminarin. In continuous cultures, the medium employed

was the basal salt solution supplemented with yeast extract (0.5 g L^{-1}), casamino acids (0.5 g L^{-1}), tributyrin (0.5 g L^{-1}), $CaCl_2$ (10^{-3} M) and glucose at varying concentrations. During investigation of the effect of specific growth rate, glucose input concentration was maintained at 2 g L^{-1} and steady states were obtained at different dilution rates, from 0.06 to 0.34 h^{-1}. For investigation of the effect of glucose concentration (from 0.5 to 4 g L^{-1}) on the two activities, a constant dilution rate of 0.04 h^{-1} was maintained and a steady state was obtained for each glucose input concentration.

Measurement of cell density and growth rates

Growth rates were determined by measuring absorbance at 600 nm (Beckman Model 25 spectrophotometer) over time. Dry weights of cells were determined directly by centrifuging a known volume of culture at 12 000 *g* for 10 min on a Sigma-202 MK centrifuge. The pellet was washed once in distilled water, then resuspended in a small volume of sterile distilled water and dried at 60 °C in a pre-weighed dry glass vial, to constant weight.

Growth conditions

In batch culture for studies of biomass and exoenzyme production (proteases and lipases) on different carbon and nitrogen sources, cells were grown aerobically in a 2-L Biolafitte fermentor (L S L-Biolafitte, Saint-Germain en Laye, France) at 22 °C and aerated with an air flow of 1 L min^{-1} and a turbine speed of 180 rpm using either R_2A or the basal salt solution supplemented with yeast extract (0.5 g L^{-1}), proteose peptone (1 g L^{-1}) for assays of protease, or with casamino acids (1 g L^{-1}) for assays of lipase. Cells were incubated, and growth was followed turbidimetrically at 600 nm, until the end of the exponential phase (usually about 6 h) for exoproteases, and until stationary phase (usually about 14 h) for lipase assays.

Samples (150 ml) were removed from the 2-L fermentor and dispensed in different 250-ml Erlenmeyer flasks. Each flask was supplemented with either a carbon or nitrogen source, and incubated at 22 °C on a gyratory water bath shaking at 150 rev min^{-1}; enzymatic activities were investigated 4 h later.

For continuous culture, all experiments were carried out in a 2-L Biolafitte fermentor with control modules for gas flow, temperature, pH, and stirrer rate. The nutritional medium was continuously added to the fermentor by a variable special pump (model Gilson Minipuls 2). A side-arm was installed to harvest the culture medium, which ensured constant culture volume. The temperature was maintained at 22 °C (±0.1 °C) and the pH was controlled at 7.1 (±0.1) by the automatic addition of NaOH (1 M) or H_2SO_4 (1 M) solutions. Steady-state conditions were seemed to be established when biomass and exoenzyme levels were constant in three consecutive determinations, at the same dilution rate or glucose uptake rate, each taken after 3 volume changes. Aeration was maintained in all cases at 1 L min^{-1}; dissolved oxygen tension was measured with a galvanic oxygen electrode (L S L-Biolafitte System).

Measurements of glucose in the culture medium

Glucose was analyzed using the glucose oxidase test. Specific glucose uptake rate ($q_{glucose}$) was expressed in units of h^{-1}, and determined according to the formula:

$$q_{glucose} = D(G_0 - G_1)/X$$

where D = dilution rate (h^{-1}), G_0 = glucose input concentration (g L^{-1}), G_1 = glucose concentration in overflow (g L^{-1}), X = cell dry mass (g L^{-1}).

Protease assay

Cell-free supernatant fluids were obtained by centrifugation of 5 ml aliquots at 12 000 *g* for 10 min at 4 °C; casein was dissolved at a concentration of 20 g L^{-1}, in a 0.1 M sodium phosphate buffer (pH 7.8) containing $CaCl_2$ at a concentration of 5 mM. 2 ml of this substrate solution were mixed with 1 ml of cell-free supernatant fluid and incubated at 40 °C for 30 min. 6 ml of a 5 g L^{-1} trichloroacetic acid solution (0.3 N) were added and the tubes were left to stand for 30 min. The precipitated protein was separated by centrifugation at 5000 *g* for 15 min. The quantity of soluble peptides in the supernatant was estimated according to Lowry et al. [11] at a wavelength of 500 nm. Units of enzyme activity (U) were calculated from a standard curve and expressed as μg of tyrosine released from casein per h at 37 °C. Specific activity was expressed as units of protease activity per mg of cell dry mass.

Lipase assay

Lipase activity was assayed according to McKellar [14] using β-naphthyl caprylate (Serva) as the substrate: 20 μl of β-naphthyl caprylate were dispersed in 1.8 ml of TES (*N*-tris(Hydroxymethyl)methyl-2-aminoethane sulfonic acid), containing 0.1 ml of taurocholate and 30 μl of water. This mixture was incubated at 37 °C for 30 min in the presence of 50 μl of the enzyme samples being studied. The β-naphtol liberated by enzyme action was transformed into a purple pigment by addition of 20 μl of freshly prepared solution of tetraazotized orthodianisidine (more commonly called 'Fast Blue') (Serva). After 5 min proteins were precipitated by 0.2 ml of trichloroacetic acid (0.72 N) and the pigment was extracted from the mixture with 5 ml of ethyl acetate. One unit of lipase activity was calculated as 1 μmol of β-napthol released per min. Specific activity was expressed as units of lipase activity per mg of cell dry mass.

Preparation of cell-free extracts

For obtention of cell-free extract, cells were harvested from the fermentor and centrifuged at 12 000 *g* for 10 min. The pellet was resuspended in 0.1 M Tris-HCl buffer, pH 7.2, and disrupted by sonication 3 times for 2 min at a frequency of 20 Kcycles s^{-1}, espaced by 30 s (MSE, 150 Watt ultrasonic desintegrator, England). After sonication, centrifugation was used to separate the particulate material and whole cells from a clear supernatant fluid, the intracellular medium.

In batch culture, enzyme productivities were defined as

the increase of lipase and protease enzymatic units obtained per mg of cell dry mass and per h. In continuous culture, the specific rate of biosynthesis of each enzyme (π), was defined as the result of the multiplication of the specific enzyme activity (U mg^{-1} of cell dry mass) and the dilution rate.

RESULTS

Batch cultures

Effects of culture conditions on proteolytic and lipolytic production by Serratia marcescens *532 S.* Studies were carried out on exoprotease and exolipase production in R_2A medium. Microorganisms grew exponentially for approximately 6 h before entering the decelerating growth phase (Fig. 1). An average maximum specific growth rate (μmax) of 0.42 h^{-1} was obtained. Cells grown in the media containing a carbohydrate, a nitrogen, and a lipid source excreted their hydrolases during the decelerating growth phase, whereas the simple carbon source was used immediately. Nevertheless, a low basal lipase activity was detected in media which did not contain lipids. Fig. 2A,B shows that the increase of enzyme activities per h was significant until 16 h of incubation, before strains entered the stationary growth phase. During this time, no observable lysis of cells occurred, and it was concluded that only viable cells secreted the two enzymes into the medium. Strain 532S was incubated in R_2A limited medium at pH 7.1 containing 1 g L^{-1} of casamino acids and 0.5 g L^{-1} of tributyrin, with different glucose concentrations (0; 0.5; 1.0; 1.5; 2.0 g L^{-1}). A proportional delay of hydrolytic activities was observed with the increasing glucose concentrations (Fig. 3A,B), indicating that lipase and protease activities were repressed by glucose concentrations.

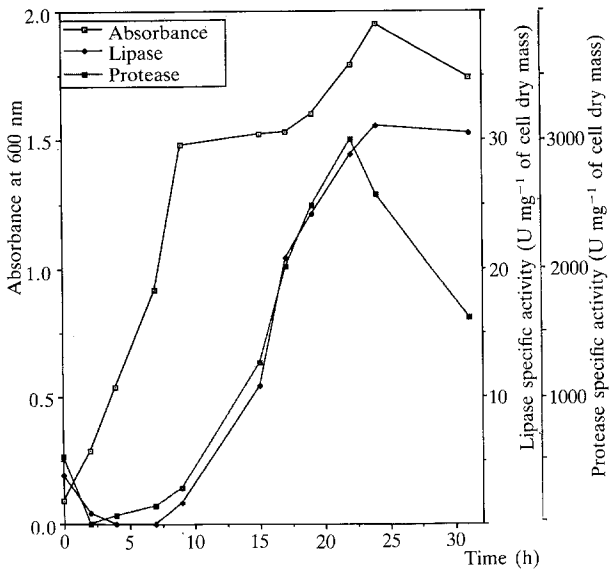

Fig. 1. Biomass, exoprotease and exolipase production by *Serratia marcescens* 532 S, grown at 22 °C in R_2A medium supplemented with 10^{-3} M of $CaCl_2$ and 0.5 g L^{-1} of tributyrin.

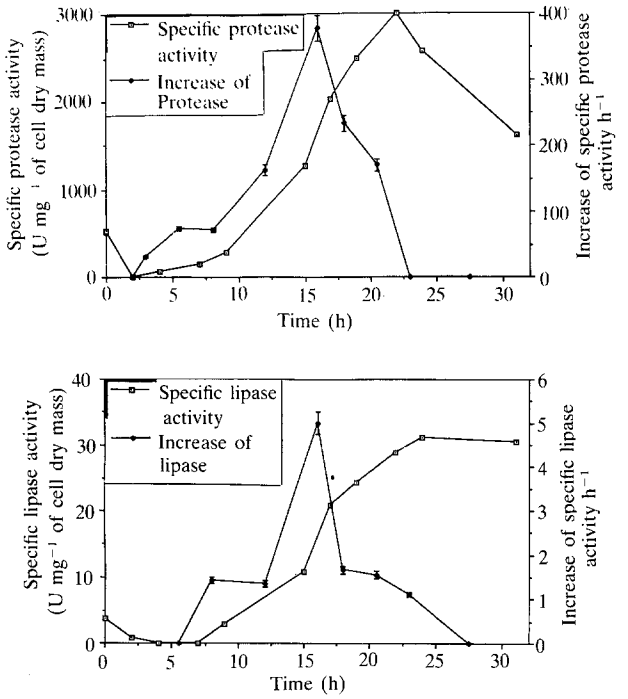

Fig. 2. A, B. Increase of exoprotease and exolipase specific activities per h, compared to their corresponding specific activities during the growth of *Serratia marcescens* 532 S on R_2A medium. The increase of specific activities were determined as described in Materials and Methods.

In an effort to determine how glucose influenced enzyme activities, a 5-h culture was used; at this time which corresponded to the exponential phase, no activity was detected. After sonication, the cell debris and the intracellular medium were added to an equal volume of a culture supernatant with known lipase and protease activity values, and subjected to protease and lipase tests. No decrease in enzyme activity was detected. These results indicate that glucose had no effect on previously synthesized enzymes. For a better understanding of these regulatory mechanisms, the effects of various other substrates were tested in defined media.

Effects of different carbon sources on exoprotease production in batch cultures. The carbon sources examined were glucose, mannitol, fructose, acetate and maltose, each added at a concentration of 1.5 g L^{-1}. These substrates were added to a culture of *Serratia marcescens* 532 S producing exoprotease. 150-ml flasks were incubated at 22 °C on a gyratory water bath shaking at 150 rev min^{-1}, for 4 h. The efficiency of specific exoprotease production per unit of biomass on the different media followed the pattern: maltose > acetate > mannitol > fructose > glucose (Fig. 4). While these simple carbon sources caused an increase in biomass production, exoprotease production was decreased with respect to the

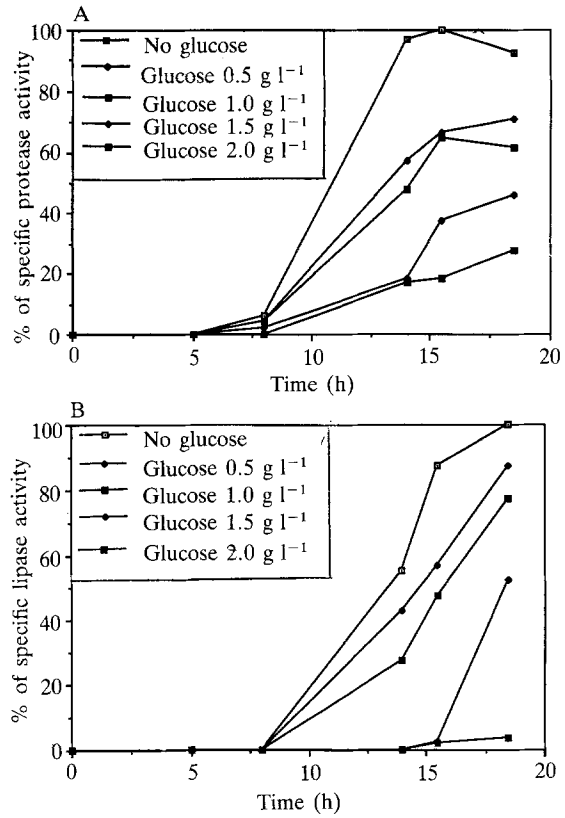

Fig. 3. A, B. Influence of initial concentrations of glucose on induction of protease and lipase activities of *Serratia marcescens*. The percentage of lipase or protease activity was measured at a concentration of respectively, 0, 0.5, 1, 1.5 and 2 g L^{-1} of glucose in the medium. Each point is the mean of at least 3 independent determinations. The specific lipase activity of 25.23 (U mg^{-1} of cell dry mass) and the specific protease activity of 1266.8 (U mg^{-1} of cell dry mass), represent the 100% values.

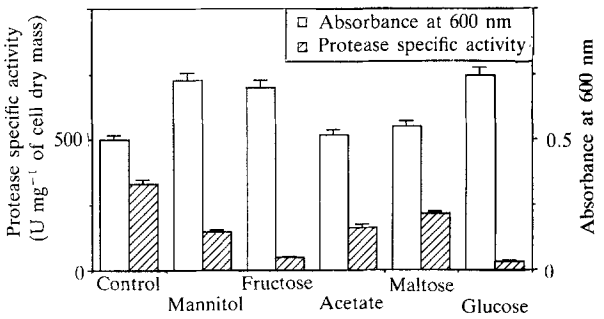

Fig. 4. Effect of different carbon sources (1.5 g L^{-1}) on protease activity and biomass production. Error bars indicate standard deviation of 3 assays.

control medium, with the greatest repression attributable to the glucose.

Effects of different nitrogen sources on exoprotease production in batch cultures. The effects were examined by supplementing cultures with 1.5 g L^{-1} of casein, casamino acids, and proteose peptone either supplied alone or augmented with different concentrations (2 and 4 g L^{-1}) of a simple inorganic nitrogen compound (NH_4Cl). Limited medium supplemented with proteose peptone and casein (proteinase substrates) increased protease specific activity. The active effect of proteose peptone was significantly affected by the addition of NH_4Cl at increasing concentrations. In the same way a simple nitrogen source such as casamino acids significantly depressed the levels of protease specific activity and increased biomass formation (Fig. 5).

Effects of different carbon and nitrogen sources on lipase production. As described previously in protease regulation studies, the media were supplemented with different carbon and nitrogen compounds. Fig. 6 shows that the simplest carbon sources easily utilisable by strain 532 S did not enhance exolipase quantity in the medium (e.g. fructose, mannitol, maltose). However, in these experiments, glycogen was the only polysaccharide which increased exolipase formation significantly for this strain. Lipids in general activated enzyme production, the most significant increase occurred in lipase activity when Tween 80 was added in the normal synthetic medium. No nitrogen compound provoked a greater enzyme formation; the nitrogen compounds tested decreased lipase formation especially NH_4Cl (at a concentration of 4 g L^{-1}), casein and tryptone. Fatty acids such as oleate, palmitate and caprylate were without effect and stearate decreased lipase specific activity.

Continuous cultures

The double reciprocal plot of glucose concentration in the fermentor versus the dilution rate of cultures of *Serratia marcescens* was investigated. A Ks (constant of saturation) of 0.0014 g L^{-1} and a maximum specific growth rate (μmax) of 0.42 h^{-1}, were found. Biosynthesis of lipase and protease activities were studied under steady-state conditions. Strain 532 S produced protease and lipase at very low dilution rates (0.06–0.1 h^{-1}) (Fig. 7). At D values between 0.06 and 0.08 h^{-1}, the level of lipase activity dropped while that of protease showed a corresponding increase. Over these values, there is with increasing D a progressive increase in the steady-state concentration of the nutrients at which growth

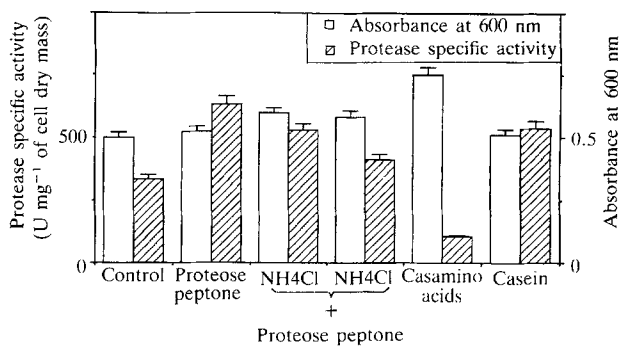

Fig. 5. Effect of different nitrogen sources (1.5 g L^{-1}) on exoprotease and biomass production by *Serratia marcescens* 532 S. Error bars indicate standard deviation of 4 assays.

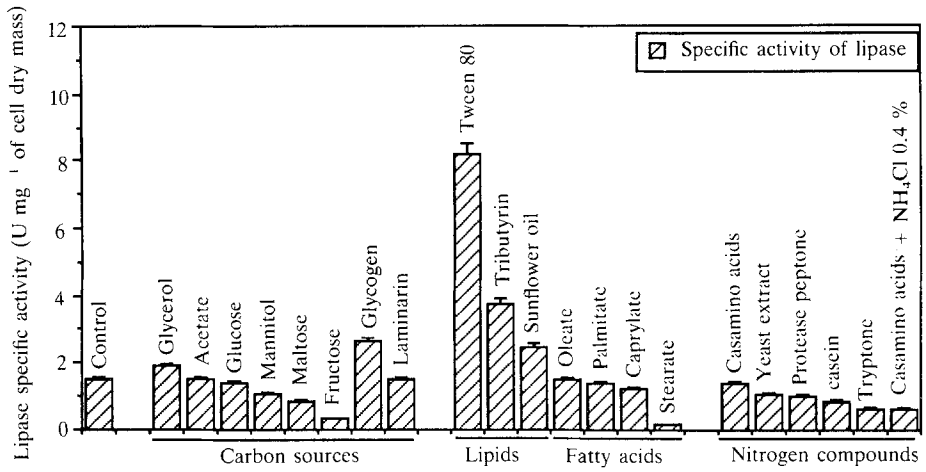

Fig. 6. Influence of different substrates (1.5 g L^{-1}) on lipase activities of strain 532 S. Error bars indicate standard deviation of 4 assays.

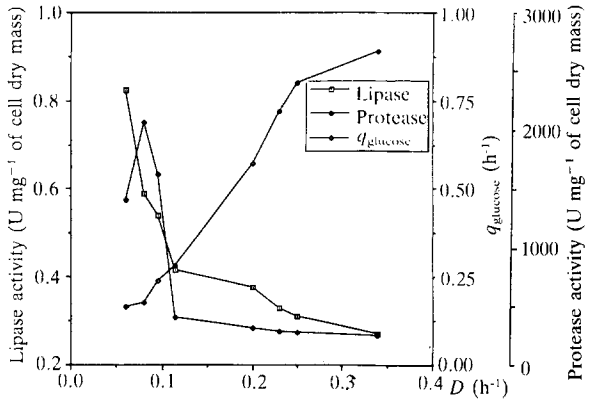

Fig. 7. Influence of the dilution rate (D), on level of steady state of exolipase and exoprotease specific activities and on specific glucose uptake rate, at constant input of casamino acids, tributyrin and glucose at levels of 0.5 g L^{-1}, 0.5 g L^{-1} and 2 g L^{-1} respectively.

occurs and hence a progressive increase in the specific glucose uptake rate (Fig. 7) and a decrease of the two hydrolase activities. Similarly, the explanation for increased lipase and protease synthesis with decreasing D values is release of catabolite repression which results from lower steady-state concentrations of the limiting carbon source and hence from decreasing intracellular pools metabolites [12]. This was confirmed when variable glucose concentrations were added to the medium at a constant dilution rate of 0.04 h^{-1} (Table 1), since a specific glucose uptake rate greater than 0.065 h^{-1} affected lipase and protease synthesis.

DISCUSSION

Serratia marcescens 532 S was isolated from an aerobic fixed-bed reactor used in wastewater treatment. This bacterium, when grown on agar plates containing glucose, proteins or lipids, expressed respectively glucidolytic, proteolytic or lipolytic activity. Results obtained in this study suggest that biosynthesis of protease and lipase, which were

TABLE 1

The effect of glucose input on growth, on the level of steady-state exolipase and exoprotease specific activities, at a constant dilution rate value of 0.04 h^{-1}

	Glucose input (g L^{-1})				
Parameter	0.5	1.0	2.0	3.0	4.0
Cell dry mass (g L^{-1})	0.52	0.61	0.65	0.70	0.75
±SD	0.06	0.05	0.03	0.03	0.06
Glucose input rate (g L^{-1} h^{-1})	0.02	0.04	0.08	0.12	0.16
Residual gluc. (g L^{-1})	ND*	ND	ND	0.007	0.395
±SD	–	–	–	0.002	0.001
$q_{glucose}$ (h^{-1})	0.038	0.065	0.122	0.171	0.192
Specific. prot. act. (U mg^{-1})	631	1495	612	357	82.25
±SD	67	77	19	12	6.23
Π Prot. (U mg^{-1} h^{-1})	25.24	59.80	24.48	14.28	3.29
±SD	0.17	0.19	0.05	0.03	0.02
Specific lip. act. (U mg^{-1})	7.90	4.77	1.15	0.71	0.57
±SD	0.40	0.63	0.07	0.12	0.05
Π lip. (U mg^{-1} h^{-1})	0.316	0.190	0.046	0.028	0.023

All results were represented as the mean ±SD of 3 or 4 separated determinations.
*ND: Not detectable.

readily excreted by *Serratia marcescens* 532 S in the culture medium, was strongly regulated at several levels.

At one level, production of the two enzymes was induced by substrates upon which they acted, and inhibited by their degradation products such as casamino acids and NH_4^+ for protease, and fatty acids for lipase. That is a typical scheme of regulation already demonstrated by several authors. Ammonia has been shown to inhibit proteolysis in other gram-negative bacteria such as *Vibrio alginolyticus* [10], *Pseudomonas aeruginosa* [20], *Aeromonas hydrophila* [19], and *Butyrivibrio fibrosolvens* [4]. Repression caused by high quantities of casamino acids may be due to the presence of

one or several amino acids which strongly affect protease production; Katchalsky et al. [9] revealed that the presence of basic poly-amino acids considerably inhibited protease production, whereas Braun and Schmitz [1] showed that leucine was an inducer of protease synthesized by *Serratia marcescens*.

The effects of glycogen and Tween 80 may be considered differently. The effect of glycogen on the yield of exolipase activity was identified by Winkler and Stuckmann [22]. They proposed that helical conformation or high branching, or both, were structural prerequisites for polysaccharides to enable them to enhance the formation of exolipase by *Serratia marcescens*. In accordance with published data, tweens significantly enhance lipase production. Tween 80 is a surface active agent which, included in a medium, lowers the interfacial tension around bacteria suspended in the medium, thereby permitting more rapid entry of desired compounds into the cell [16, 17, 18].

A second regulation level on the production of the two enzymes was the catabolite glucose repression detected at high bacterial glucose uptake rates. When concentrations of glucose were higher than 0.5 g L^{-1} in batch cultures, very low levels of lipase and protease activities were detected. The same phenomenon was observed under continuous culture conditions, at glucose input rates higher than 0.04 g L^{-1} h^{-1}. In continuous cultures at D values lower than 0.08 h^{-1}, it appeared that protease and lipase patterns between enzyme production and growth rate were not exactly identical. This indicated that the characteristics of the regulatory mechanism involved were not the same as explained according with other work [21]. Indeed, when this strain was grown in continuous cultures, in the presence of casamino acids, tributyrin and glucose, the organism exhibited a sharp peak of protease activity at a D value of 0.08 h^{-1}; this can be interpreted by a balance between casamino acids induction and glucose catabolite repression. At low D values, with low concentrations of casamino acids (0.5 g L^{-1}) in the medium, the degree of induction limited the amount of enzyme synthesized, and D values greater than 0.08 h^{-1} led to a progressive increase in catabolic repression of the enzyme synthesis. The same conclusion was demonstrated in a previous paper [3] concerning studies of amidase synthesis by *Pseudomonas aeruginosa* and its various mutants. However, lipase synthesis appeared to be regulated only by glucose catabolic repression and induction by tributyrin.

Our research confirms that protease and lipase production by *Serratia marcescens* 532 S, grown in a culture medium with glucose, proteins and lipids, is strongly affected by induction, catabolite repression and end-product inhibition. Thus, in an aerobic fixed-bed reactor this microorganism will act only as a glucidolytic bacterium at high dilution rates of wastewater and as a generalist bacterium (with simultaneous glucidolytic, proteolytic and lipolytic activities) at low dilution rates.

ACKNOWLEDGEMENTS

We are grateful to Doctor G. Bacquet and Doctor J.C. Joret (Compagnie Générale des Eaux, Maisons-Laffitte, France) for discussions and to G. Raval for technical assistance.

REFERENCES

1 Braun, V. and G. Schmitz. 1980. Excretion of a protease by *Serratia marcescens*. Archiv. Microbiol. 124: 55–61.
2 Bromke, B.J. and J.M. Hammel. 1979. Regulation of extracellular protease formation by *Serratia marcescens*. Can. J. Microbiol. 25: 47–52.
3 Clarke, P.H., M.A. Houldsworth and M.D. Lilly. 1968. Catabolite repression and the induction of amidase synthesis by *Pseudomonas aeruginosa* 8602 in continuous cultures. J. Gen. Microbiol. 51: 225.
4 Cotta, M.A. and R.B. Hespell. 1986. Proteolytic activity of the ruminal bacterium *Butyrivibrio fibrisolvens*. Appl. Environ. Microbiol. 52: 31–58.
5 Dwight, A.H., P.N. Sauruger, G.M. Ihler and M. Benedik. 1988. Genetic analysis of extracellular proteins of *Serratia marcescens*. J. Bacteriol. 170: 4141–4146.
6 Grimont, P.A.D., F. Grimont and H.L.E. Dulong de Rosnay. 1977. Characterization of *Serratia marcescens, S. liquefaciens, S. plymuthira* and *S. marinorubia* by electrophoresis of their proteinases. J. Gen. Microbiol. 99: 301–310.
7 Heller, K.B. 1979. Lipolytic activity copurified with the outer membrane of *Serratia marcescens*. J. Bacteriol. 140: 1120–1122.
8 Henriette, C., E. Petitdemange, G. Raval and R. Gay. 1991. Mercuric reductase activity in the adaptation to cationic mercury, phenyl mercuric acetate and multiple antibiotics of a Gram-negative population isolated from an aerobic fixed-bed reactor. J. Appl. Bacteriol. 71: 439–444.
9 Katchalsky, E., S. Bichowski, L. Lomnitski and B.E. Volcani. 1953. The action of some water-soluble poly-amino acids on the bacteria. Biochemistry 55: 671–680.
10 Long, S., M.A. Mothibelli, F.T. Robb and D.R. Woods. 1981. Regulation of extracellular alkaline protease activity by histidine in a collagenolytic *Vibrio alginolyticus* strain. J. Gen. Microbiol. 127: 193–199.
11 Lowry, O.H., V.J. Rosebrough, A.L. Fara and R.J. Randol. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
12 Matin, A. 1981. Regulation of enzyme synthesis as studied in continuous culture. In: Continuous Culture of Cells (Calcott, P.H. ed.), Vol II. pp. 69–95. CRC Press.
13 McKellar, R.C. 1982. Factors influencing the production of extracellular proteinase by *Pseudomonas fluorescens*. J. Appl. Bacteriol. 53: 305.
14 McKellar, R.C. 1986. A rapid colorimetric assay for the extracellular lipase of *Pseudomona fluorescens* B52 using β-naphthyl caprylate. J. Dairy Res. 53: 117–127.
15 McKellar, R.C. and H. Cholette. 1985. Stimulation by calcium of the formation of active extracellular proteinase by *Pseudomonas fluorescens* spp 1. J. Dairy Science 68: 3216–3223.
16 Nahas, E. 1988. Control of lipase production by *Rhizopus oligosporus* under various growth conditions. J. Gen. Microbiol. 134: 227–233.
17 Ota, Y., S. Miyairi and K. Yamada. 1968a. Sterol requirements of the lipase production by *Candida cylindracea*. Agric. Biol. Chem. 3: 1476–1478.
18 Ota, Y., M. Sukuki and K. Yamada. 1968b. Lipids and related substances inducing the lipase production by *Candida paralipolytica*. Agric. Biol. Chem. 32: 390–391.
19 Pansare, A.C., V. Venugopal and N.F. Lewis. 1985. A note

of nutritional influence on extracellular protease synthesis in *Aeromonas hydrophila*. J. Appl. Bacteriol. 58: 101–104.

20 Whooley, M.A., J.A. O'Callaghan and A.J. McLoughlin. 1983. Effect of substrate on the regulation of exoprotease production by *Pseudomonas aeruginosa* ATCC 10145. J. Gen. Microbiol. 129: 981–988.

21 Wiersma, M. and W. Harder. 1978. A continuous culture study of regulation of extracellular protease production in *Vibrio* SA1. Antonie Van Leeuwenhoek. 44: 141–155.

22 Winkler, U.K. and M. Stuckmann. 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J. Bacteriol. 138: 663–670.