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Optimization of glycosidases production by *Pseudoalteromonas issachenkonii* KMM 3549^T

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Aims: The present work aimed to design an optimized medium to yield a higher production of glycosides by *Pseudoalteromonas issachenkonii* KMM 3549^T.

Methods and Results: Higher levels of fucoidan hydrolase, alginase, laminaranase and *b*-N-acetylglucosaminidase production were obtained with peptone concentrations ranging from 2·5 g l^{-1} to 10 g l^{-1}, while the presence of both yeast extract and glucose did not affect enzyme production. The activity of fucoidan hydrolase and laminaranase increased up to 4·83 μM h^{-1} mg^{-1} and 19·23 μM h^{-1} mg^{-1} protein, respectively, in growth media containing xylose (1·0 g l^{-1}), laminarin (0·5 g l^{-1}) or alginate (0·5 g l^{-1}), and production of *b*-N-acetylglucosaminidase substantially increased in the presence of fucoidan (0·5 g l^{-1}) or galactose (1 g l^{-1}). All polysaccharides tested in concentrations of 0·5 g l^{-1} fucoidan and 0·2 g l^{-1} fucose induced production of alginase (up to 5·06 μM h^{-1} mg^{-1} protein).

Conclusions: The production of glycosidases is not only stimulated by the presence of algal polysaccharides, but may also be stimulated by monosaccharides (e.g. xylose).

Significance and Impact of the Study: The production of glycosidases by *Pseudoalteromonas issachenkonii* KMM 3549^T was significantly improved by using a simple nutrient medium containing peptone (2·5 g l^{-1}) and xylose (5·0 g l^{-1}) in 100% natural seawater.

INTRODUCTION

Microbial communities residing on the surfaces of macroalgae are characterized by wide-ranging physiological adaptations and a high level of metabolic activity (Cho and Azam 1988; Warren 1996; Jaffray *et al.* 1997; Holmström and Kjelleberg 1999). Recently we described the symbiotrophic associations of the marine aerobic gamma *Proteobacteria*, which are able to degrade the thallus of the brown algae *Fucus evanescens* (Ivanova *et al.* 2002a). A member of these associations assigned to the new species *Pseudoalteromonas issachenkonii* KMM 3549^T (Ivanova *et al.* 2002b) produced a number of glycosidases, namely fucoidan hydrolase, laminaranase, alginase, pustulanase, β-glucosidase, β-galactosidase, β-N-acetylglucosaminidase and β-xylosidase. This wide range of hydrolytic enzymes catalyse the hydrolysis of the main polysaccharides, e.g. alginic acids, cellulose, laminarans, and fucoidans of brown alga (Warren 1996). Laminarins (1,3-β-D-glucans) and its analogues were found in fungal cell walls, plants and lichens, while alginic acids and fucoidans were never detected in terrestrial sources. Fucoidans comprise an array of sulphated homo- and heteropolysaccharides composed of α-(1(r)2)- and/or α-(1(r)3)-linked L-fucose residues (Patankar *et al.* 1993; Bilan *et al.* 2002) and also contain residues of galactose, mannose, xylose and glucuronic acid. The proportion of sulphated homo- and heteropolysaccharides as well as mono- and disaccharides varies greatly depending on both the species and geographical origin of the brown algae (Nishino *et al.* 1991; Beress *et al.* 1993; Zvyagintseva *et al.* 1999). Because of such diversity, precise structures of fucoidans remain unclear. During the last decade studies of fucoidan structures received increasing attention as a result of their pharmacological

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importance (antibacterial, antiviral, antitumour, immunosuppressive, antipeptic and anticoagulant, etc. activities (Painter *et al.* 1965; McClure *et al.* 1992; Beress *et al.* 1993). However, the employment of these polysaccharides in biotechnological applications is limited for a few reasons. One of these is the lack of hydrolytic enzymes capable of decomposing algal polysaccharides into small fragments to facilitate the elucidation of their structure, function and biological activities. Microbial glycosidases that act on brown algae polysaccharides including fucoidans might be a promising tool for medical biotechnology. Only a few microbial fucoidanases have been reported until recently, e.g. from *Vibrio* sp. N-5 with relatively modest, specific activity (Furukawa *et al.* 1992) and from *Flavobacterium* sp. SA002 with high, specific activity (Sakai *et al.* 1997).

This study is a part of the research program initiated in the Pacific Institute of Bio-organic Chemistry for the search of microbial producers of glycosidases. Earlier we showed that marine bacteria of the genera *Flavobacterium Cytophaga* were capable of degrading fucoidan (Bakunina *et al.* 2002). Here we present the results of the evaluated components of the culture medium likely to affect fucoidan hydrolase, laminaranase, alginase and β-N-acetylglucosaminidase production and define the optimized composition of culture medium for the production of glycosides by *Pseudoalteromonas issachenkonii* KMM 3549^T.

MATERIALS AND METHODS

Bacterial strain and growth condition

The producer strain *Pseudoalteromonas isshachenkonii* KMM 3549^T was isolated from the degraded thallus of brown algae *F. evanescens* in 1999 as described elsewhere (Ivanova *et al.* 2002a), and initially was grown on marine agar 2216 (Difco Laboratories, Detroit, MI) and on plates with medium B, containing 0·2% (w/v) Bacto Peptone (Difco), 0·2% (w/v) casein hydrolysate (Merck), 0·2% (w/v) Bacto Yeast Extract (Difco), 0·1% (wt/vol.) glucose, 0·02% (w/v) KH_2PO_4, 0·005% (w/v) $MgSO_4 \cdot 7H_2O$ and 1·5% (w/v) Bacto Agar (Difco) and 50% (v/v) each of natural seawater and distilled water at pH 7·5–7·8. The strains were maintained on the same semisolid B medium in tubes under mineral oil at 4 °C, and stored at −80 °C in marine broth (Difco) supplemented with 20% (v/v) glycerol.

Selection of growth media

To select the optimal growth medium, the growth rate, protein and enzyme activities yields were compared by using the media defined in Tables 1 and 2. All experiments were carried out in 0·5-l Erlenmeyer flasks containing 100 ml of culture medium on the shaker (170 r.p.m.) for 20 h at 28 °C. The inoculum size used in all fermentation experiments was 5% (v/v) of 20-h old (O.D.$_{650}$ 1·0) culture grown on medium B. All fermentations were conducted in duplicate, with two repeats of each fermentation run, and data were analysed with the ANOVA program using Statistica software (rel 4·3 S, StatSoft Inc, Ubi TechPark, Singapore 1993). Growth was monitored spectrophotometrically (O.D.$_{650}$).

Table 1 Effect of peptone, yeast extract and glucose on fucoidan hydrolase production by *Pseudoalteromonas issachenkonii* KMM 3549^T

Peptone (5·0 g l^{-1})	Yeast extract (2·0 g l^{-1})	Glucose (1·0 g l^{-1})	Protein (mg ml^{-1})	Specific enzyme activity* (μM h^{-1} mg^{-1} protein)
+	+	–	0·77 ± 0·09	0
–	+	–	0·21 ± 0·01	0
+	–	+	0·21 ± 0·01	1·77 ± 0·17
–	–	+	0	0
+	–	–	0·28 ± 0·01	1·56 ± 0·14
+	+	+	0·84 ± 0·10	0

*Values are averages of three measurements ± standard deviations.

Crude enzyme extraction

After fermentation cells were collected by centrifugation for 30 min (5000 g) at 4 °C and disrupted by ultrasonication at 20 kHz for 20 s (three times) in 10 ml of 50 mM phosphate buffer, pH 7·2. Suspension was centrifuged at 12000 × *g* for 20 min to remove cell debris. The supernatant was used as the crude enzyme solution.

Enzyme assay

Fucoidan obtained from the brown alga *F. evanescens* (Zvyagintseva *et al.* 1999), laminaran from the brown alga *Laminaria cichorioides* (Zvyagintseva *et al.* 1994), kappa-carrageenan kindly provided by Dr E.L. Nazarenko, and alginate, agarose and β-N-Ac-glucosaminide obtained from Serva Corp. (Wichita Falls, TX, USA) were used as substrates for determining the activities of fucoidan hydrolase, laminaranase, alginase and β-N-Ac-glucosaminidase, respectively. Enzyme activities were measured by colourimetric analysis of reducing sugars (Nelson 1944) and were expressed as the amount of enzyme that liberated 1 μmol of correspondent substrate per 1 mg of protein. The amount of protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. One unit of glycanase activity was defined as the amount of enzyme that liberated 1 μmol of corresponding monosaccharides per 1 h.

RESULTS

In the initial experiments we studied the effect of peptone, yeast extract and glucose on the production of the fucoidan

Table 2 Effect of different concentrations of peptone, mono- and polysaccharides on protein biosynthesis and the specific enzyme activities of glycosidases produced by *Pseudoalteromonas issachenkonii* KMM 3549^T

Culture medium ingredients (g l^{-1})	Protein (mg ml^{-1})	Specific enzyme activity* (μM h^{-1} mg^{-1} protein)			
		Fucoidan hydrolase	Laminaranase	Alginase	β-N-Ac-Glucosaminidase
Peptone, 1	0.1	0	0	10·49 ± 0·9	4·2 ± 0·003
Peptone, 2·5	0·17 ± 0·01	1·76 ± 0·15	2·49 ± 0·10	4·46 ± 0·45	3·6 ± 0·003
Peptone, 7·5	0·46 ± 0·05	0·95 ± 0·07	2·12 ± 0·08	1·58 ± 0·15	4·9 ± 0·004
Peptone, 10·0	0·5 ± 0·02	1·17 ± 0·09	1·82 ± 0·07	1·8 ± 0·18	3·0 ± 0·002
D-Fucose, 0·2	0·17 ± 0·01	3·83 ± 0·19	3·27 ± 0·13	5·06 ± 0·03	2·5 ± 0·002
D-Galactose, 1·0	0·23 ± 0·02	3·75 ± 0·018	3·36 ± 0·13	4·45 ± 0·04	3·5 ± 0·002
D-Rhamnose, 1·0	0·14 ± 0·01	4·66 ± 0·22	1·75 ± 0·08	5·04 ± 0·025	2·7 ± 0·002
D-Xylose, 0·5	0·14 ± 0·01	1·91 ± 0·15	0·27 ± 0·02	3·84 ± 0·03	2·5 ± 0·002
D-Xylose, 1·0	0·17 ± 0·02	4·83 ± 0·38	3·98 ± 0·36	4·67 ± 0·04	2·3 ± 0·002
D-Xylose, 5·0	0·08 ± 0·01	6·18 ± 0·30	1·46 ± 0·09	4·88 ± 0·04	2·0 ± 0·001
D-Galactose, 0·5 + D-Rhamnose, 0·5 + D-Xylose, 0·5	0·16 ± 0·01	3·36 ± 0·22	0·54 ± 0·09	4·37 ± 0·05	2·4 ± 0·003
Agarose, 1·0	0·17 ± 0·01	0	0·2 ± 0·04	5·44 ± 0·04	1·2 ± 0·001
Fucoidan, 1·0	0·45 ± 0·07	0·49 ± 0·10	3·74 ± 0·05	2·66 ± 0·18	4·9 ± 0·004
Laminarin, 1·0	0·3 ± 0·01	1·95 ± 0·29	15·05 ± 0·15	3·89 ± 0·26	2·9 ± 0·002
Alginate, 1·0	0·2 ± 0·01	1·99 ± 0·31	19·23 ± 0·02	5·78 ± 0·38	2·4 ± 0·002
Kappa-Carrageenan, 1·0	0·19 ± 0·01	0	1·79 ± 0·14	5·42 ± 0·36	1·8 ± 0·001

*Values are averages of three measurements ± standard deviations.

hydrolase by *Pseudoalteromonas issachenkonii* KMM 3549^T (Table 1). The results obtained showed that even though the bacteria grew well on the culture media supplemented with either peptone, yeast extract and glucose or peptone and yeast extract, they did not produce the fucoidan hydrolase. Further, neither the presence of yeast extract nor glucose alone had an effect on enzyme production. Only peptone and a combination of peptone and glucose were essential for the synthesis of fucoidan hydrolase.

The effect of different peptone concentrations on fucoidan hydrolase, laminaranase, alginase and β-N-acetylglucosaminidase production by *P. issachenkonii* was determined and the data are shown in Table 2. Peptone at a concentration of 2·5 g l^{-1} was optimal for the synthesis of all enzymes studied, while peptone at 1 g l^{-1} and 7·5 g l^{-1} significantly increased the specific activities of alginase (up to 10·49 μM h^{-1} mg^{-1} protein) and β-N-acetylglucosaminidase (up to 0·049 μM h^{-1} mg of protein), respectively.

The effect of a range of carbohydrates (i.e. fucose, xylose, rhamnose and galactose) as components of polysaccharides and that of polysaccharides (fucoidan, laminaran, alginate and agar) as constituents of brown and red algal cell walls on enzyme production was also determined (Table 2). The results revealed that the presence of individual carbohydrates was stimulatory for enzyme production to differing extents. In contrast, the addition of fucose, xylose, rhamnose and galactose together did not affect enzyme production. Specifically, alginase production was induced by fucose and rhamnose, yielding the specific enzyme activities of 5·06 μM h^{-1} mg and 5·04 μM h^{-1} mg of protein, respectively. The production of β-N-acetylglucosaminidase was increased by galactose a specific activity up to 0·035 μM h^{-1} mg of protein.

The stimulatory effect of xylose in a concentration of 1·0 g l^{-1} on fucoidan hydrolase and laminaranase production was the most prominent. Therefore, we studied the effect of various concentrations of xylose on enzyme production (Table 2). Interestingly, 5 g l^{-1} of xylose was optimal for production of fucoidan hydrolase and alginase, with yields of specific activities up to 6·18 μM h^{-1} mg and 4·88 μM h^{-1} mg^{-1} protein, respectively, but not for laminaranase production.

Finally, the effect of algal polysaccharides on the production of glycosidases was studied. The stimulatory effect of laminarin and alginate was remarkable. The yields of specific activities of laminaranase and alginase increased by up to 15·05–19·23 μM h^{-1} mg^{-1} and 3·89–5·78 μM h^{-1} mg^{-1} protein, respectively. However, the addition of either of five algal polysaccharides, namely agarose, fucoidan, laminaran, alginate or carrageenan, did not result in a significant stimulation of fucoidan hydrolase production.

DISCUSSION

As the optimization of the growth environment is important to achieving maximal enzyme production, the evaluation of the effects of components of culture medium is of particular interest.

The analysis of the data obtained as well as of previous findings (Aoki *et al.* 1990; Furukawa *et al.* 1992; Jaffray *et al.* 1997) suggest that peptone and some polysaccharides (i.e. in this study laminarin and alginate) are the most sufficient components of the nutrient media useful for bacterial growth and glycosidases production. The yields of specific enzyme activities of *P. issachenkonii* KMM 3549^T were 2–3-fold greater or comparable to those of fucoidan hydrolase and laminarinase reported earlier (Furukawa *et al.* 1992; Sova *et al.* 1994; Sakai *et al.* 1997) and comparable to that of alginase lyase produced by *Alteromonas* sp. (Sawabe *et al.* 1997). Further, this study revealed that the replacement of these polysaccharides to xylose gave a similar positive effect on glycosidases production. The profile of optimum conditions for glycosidases production by *P. issachenkonii* KMM 3549^T led to the conclusion that increased enzyme activities (as considerable as 3–5-fold) were obtained on a simple nutrient medium containing peptone (2·5 g l^{-1}) and xylose (5·0 g l^{-1}) in 100% natural seawater. Therefore we suggest that xylose might be used as an alternative cheap carbon source for the production of some glycosidases.

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