Journal of Industrial Microbiology & Biotechnology (2000) 24, 51–57
© 2000 Society for Industrial Microbiology 1367-5435/00 $15.00
www.nature.com/jim

Production of ethanol from mannitol by *Zymobacter palmae*

SJ Horn[1], IM Aasen[2] and K Østgaard[1]

[1]*Department of Biotechnology, The Norwegian University of Science and Technology NTNU, N-7491 Trondheim, Norway;*
[2]*SINTEF Applied Chemistry, N-7465 Trondheim, Norway*

Extracts from brown seaweeds could possibly be fermented to ethanol, particularly seaweeds harvested in the autumn, which contain high levels of easily extractable laminaran and mannitol. Few microorganisms are able to utilise mannitol as a substrate for ethanol production and *Zymobacter palmae* was tested for this purpose. Bacterial growth as well as ethanol yield depended on the amount of oxygen present. Strictly anaerobic growth on mannitol was not observed. At excessive aeration, a change in the fermentation pattern was observed with high production of acetate and propionate. Under oxygen-limiting conditions, the bacteria grew and produced ethanol in a synthetic mannitol medium with a yield of 0.38 g ethanol (g mannitol)$^{-1}$. *Z. palmae* was also successfully applied for fermentation of mannitol from *Laminaria hyperborea* extracts. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 51–57.

Keywords: *Zymobacter palmae*; mannitol; ethanol; biofuel; seaweed

Introduction

Brown seaweeds may have a high content of laminaran and mannitol. Generally, the chemical composition of brown algae varies considerably between species, throughout the year and between habitats. Brown algae exposed to seasonal changes usually accumulate mannitol and laminaran in the light season (spring to autumn), and consume these storage carbohydrates in the dark season [5]. Prominent along the Norwegian coast are the Laminarales which store most of the carbohydrates in the fronds. For autumn fronds of *Laminaria hyperborea*, the mannitol and laminaran content may be as high as 25% and 30% of the dry weight, respectively [7]. This high carbohydrate content makes brown seaweeds a potential source for production of liquid fuels such as ethanol. These storage sugars can easily be extracted from milled seaweed at low pH and high temperature [13].

Laminaran is essentially a linear polysaccharide of (1 → 3)-β-D-glucopyranose in which the chain terminates with D-mannitol. Low levels of branching via (1 → 6)-β-glucosidic linkages may occur [10]. β-(1 → 3)-glucanases are relatively widespread, and many microorganisms can hydrolyse laminaran to its glucose monomer, which is a good substrate for fermentation. Mannitol, the sugar alcohol corresponding to mannose, on the other hand is not readily fermented. It is oxidised to fructose by mannitol dehydrogenase, a reaction that generates NADH. Regeneration of NAD^+ requires oxygen (active electron transport chain) or transhydrogenase, which converts NADH to NADPH. Thus, many microorganisms are not able to carry out strictly anaerobic fermentation of mannitol [18].

Both yeast and bacteria are potential organisms for ethanol production. Yeast lack transhydrogenase [18], and experiments done with *Saccharomyces* concluded that pure anaerobic growth on mannitol was not possible [14]. Thus, fermentation of sugar alcohols by yeast requires a supply of oxygen. This subject has been thoroughly investigated in connection with ethanol production from xylose, where metabolism of the xylitol intermediate depends on oxygen [8]. Procaryotes, on the other hand, usually possess transhydrogenase [4,6] and should be able to ferment mannitol under truly anaerobic conditions.

Ethanol-fermenting bacteria include the genera *Zymomonas* and *Saccharobacter*, but they have not been reported to ferment mannitol. A novel ethanol fermenting bacterium isolated from palm sap, *Zymobacter palmae*, is reported to be facultatively anaerobic and able to ferment hexoses, α-linked di- and tri-saccharides, and sugar alcohols including mannitol [11]. Dobson and Franzmann [2] concluded in 1996 that the genus *Zymobacter* was closely related to the genus *Halomonas*, and proposed that *Zymobacter* should be transferred to the family *Halomonadaceae*.

The purpose of this work was to evaluate *Z. palmae* as a potential organism to carry out the fermentation of mannitol in seaweed extract to ethanol. Its abilities for growth and ethanol production in synthetic mannitol medium were investigated under different oxygen regimes. Experiments were also run with laminaran and glucose as substrate. Finally, ethanol production was examined in a culture medium of seaweed extract.

Materials and methods

Microorganism

The bacterium *Zymobacter palmae* T109 (ATCC 51623) was used in all the experiments. The stock culture was maintained at –80°C in 15% glycerol.

Medium

Shake bottle experiments were carried out in the medium (g L^{-1}): K_2HPO_4, 7.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; yeast extract (as a source of nicotinic acid), 0.5; and mannitol, 20. The pH was adjusted to 6.0 with HCl before autoclaving the medium (121°C, 20 min). Preculture

Correspondence: SJ Horn, Dept of Biotechnology, The Norwegian University of Science and Technology NTNU, N-7491 Trondheim, Norway. E-mail: svein@chembio.ntnu.no
Received 27 June 1999; accepted 23 September 1999

medium was the same except that it contained 5.0 g L^{-1} yeast extract.

For the fermentor experiments, the following media were used: (1) medium with 20 g L^{-1} carbon source (g L^{-1}): KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 2.5; yeast extract, 3.0; (2) medium with 40 g L^{-1} carbon source (g L^{-1}): KH_2PO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 0.4; $(NH_4)_2SO_4$, 5.0; yeast extract, 3.0. The carbon source was either glucose or mannitol, or a mixture of both, and the concentrations were chosen to mimic the seaweed extract concentrations. The fermentors containing the medium were sterilised at 121°C for 30 min. The carbon source was autoclaved separately.

The seaweed medium was prepared by extracting fresh *Laminaria hyperborea* fronds with water at pH 2.0 and 65°C according to Percival and McDowell [5]. One kilogram wet weight fronds was extracted in 1 L tap water for 1 h, yielding extracts with approximately 20 g L^{-1} of both mannitol and laminaran. The extractions were carried out in 3-L fermentors (Applicon, Schiedam, Netherlands) and the final extracts were stored at –20°C. The pH was adjusted to 6.0 with HC1 before autoclaving the medium (121°C, 20 min).

Culture conditions and experiments

All shake bottle experiments with mannitol medium were performed at 30°C in 50-ml Pyrex bottles, sealed or open. The bottles were inoculated with 2.5% (v/v) stock culture and incubated on a reciprocal shaker (180 cycles min^{-1}, amplitude 5 cm).

The seaweed extract experiments were carried out in 250-ml Erlenmeyer flasks. The flasks were inoculated with the pellet from a culture grown on mannitol medium, giving an initial biomass concentration of about 23 g L^{-1}. The flasks were incubated on a reciprocal shaker at 30°C.

All other experiments were carried out in 3-L fermentors (Applicon) with a 1-L working volume. The pH was automatically kept at pH 6.0 by controlled addition of 3 M NaOH; and the temperature was maintained at 30°C, reported to be the optimum conditions for growth [11]. CO_2 in outlet gas (Binos 100.2 M gas analyser, Hanau, Germany), stirrer speed and dissolved oxygen were logged automatically. The fermentors were inoculated with 30–50 ml of a preculture with a cell density of about 0.7 g L^{-1}.

Determination of K_La

The oxygen transfer coefficient, K_La, was determined by measuring the oxygen concentration (Rosemount, Oxynos 100 gas analyser, Hanau, Germany) in the outlet airflow from an active stationary phase culture of *Z. palmae*. Defining OUR as the oxygen uptake rate and OTR as the oxygen transfer rate, under steady state conditions, OUR = OTR = $K_La \times (C_S - C)$, where C_S is the dissolved oxygen (DO) concentration in air-saturated medium, and C is the actual oxygen concentration in the medium during the fermentation.

Sampling and analytical methods

Samples were taken aseptically, centrifuged ($9000 \times g$, 3 min), and the supernatants were stored frozen at –20°C until analysed.

Growth was quantified by measuring the optical density (OD) at 660 nm using water as reference. Cell dry weight was determined by centrifugation (25 ml broth, $3500 \times g$, 10 min), washing in distilled water and heating to constant weight under IR-lamps. A correlation factor, dry weight = $OD_{660} \times 0.345$, was calculated as an average value from all the measured samples.

Mannitol, glucose, ethanol and organic acids were quantified by high performance liquid chromatography (HPLC) of samples filtered through 0.22-μm Millipore filter Type GS. The analyses were conducted with a Shimadzu LC.9A liquid chromatograph equipped with a Shimadzu column oven CTO-6A, a Shimadzu autoinjector SIL-6A, a Shimadzu refractive index detector RID-6A, a Shimadzu C-R4A integrator, Biorad Aminex HPX-87H column (300×7.8 mm) and Biorad Micro-Guard cation H^+ refill cartridge guard column. Other conditions included: effluent, filtered and degassed 50 mm H_2SO_4; flow rate, 0.6 ml min^{-1}; and column temperature 45°C. Calibration of the method against reference compounds was performed within a standard error of ±1.5%.

Results and discussion

Glucose as substrate

Preliminary experiments showed that *Z. palmae* could not utilise laminaran directly as a carbon source (results not included). However, the laminaran monomer, glucose, could be utilised [11]. Hydrolysis of laminaran may be achieved in a co-culture where laminaran is hydrolysed by another organism. Fermentations with glucose as substrate will provide useful information of relevance for utilisation of laminaran, as well as give indications of the organism's ability to produce ethanol, in comparison to other ethanol-producing microorganisms. Results from an anaerobic and an aerated batch fermentation are presented in Figure 1. In the anaerobic fermentation, a sharp decrease of CO_2-emission rate (CER) was observed at 14.7 h, while in the aerated fermentation the maximum CER at 13.8 h was maintained until 16 h, when glucose presumably was exhausted. In Figure 1 this halt in the increase of CER is reflected as a shift in the accumulated CO_2 curve from a roughly exponential to a more linear phase. Thus, in both fermentations there seemed to be a halt in the increase of CER occurring before glucose was exhausted. These maximum CERs probably coincided with the halt in cell growth, although this was not clearly documented in this particular experiment due to infrequent sampling. However, glucose consumption and ethanol production continued until glucose was depleted.

Compared to growth in the anaerobic reactor, growth during aeration was considerably faster with twice the cell yield, while the ethanol yield was slightly lower (Table 1). Maximum biomass concentrations, derived from OD measurement, were 0.75 and 0.90 g L^{-1} for the anaerobic and aerated reactors, respectively. After glucose depletion, accumulation of acetate and propionate was observed in the aerated fermentation (results not shown), concomitant with an increase in NaOH addition rate. Increasing NaOH addition was also observed in the anaerobic fermentation, but here no organic acids could be detected. In the aerated fermentation, biomass (48% of dry weight assumed to be carbon), CO_2, ethanol, acetate and propionate accounted for

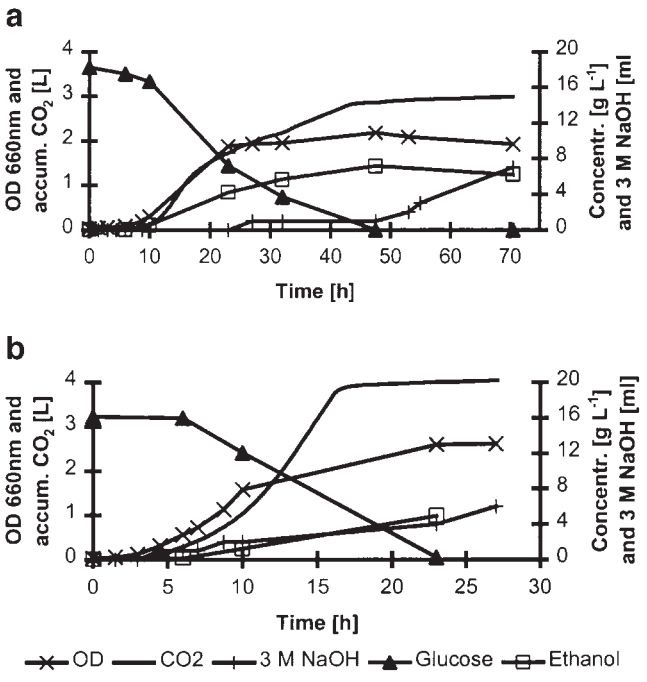

Figure 1 Glucose metabolism: optical density, accumulated CO_2, consumption of 3 M NaOH, glucose and ethanol concentrations in (a) fermentor sparged with 0.05 L N_2 (L medium min)$^{-1}$ and (b) fermentor aerated with 0.20 L air (L medium min)$^{-1}$ (OTR = 3.56 mmol O_2 L^{-1} h^{-1}).

106% of the carbon in the consumed glucose after 23 h. Similar calculations for the anaerobic fermentation after 47.5 h accounted for only 76% of the glucose carbon. The mismatch in the carbon balance, and the late increase in base consumption without accumulation of organic acids, could indicate formation of a new metabolite that was not detected by HPLC.

After inoculation the oxygen concentration in the aerated reactor (Figure 1b) decreased below the limit of detection within 4 h, leading to oxygen-limiting conditions for most of the fermentation. A separate aerobic experiment, with 40 g L^{-1} glucose in the medium and with the dissolved oxygen concentration (DO) maintained at 35% of saturation, resulted in a final biomass concentration of 1.2 g L^{-1}. Also in this case the biomass maximum was reached before exhaustion of glucose. Thus, oxygen limitation was not the cause of the halt in cell growth. However, the maximal growth rate was much higher in the aerobic fermentation (Table 1), showing a positive effect of oxygen on growth rate. No ethanol was produced, but propionate accumulated, reaching a concentration of 13 g L^{-1}.

Figure 1 shows that *Z. palmae* is able to grow on glucose both under aerobic and anaerobic conditions, confirming the observation of Okamoto *et al* [11] that the bacterium is facultatively anaerobic. From Table 1 it is seen that μ and Y_{XS} were lower under anaerobic conditions, while Y_{PS} was a little higher than in the aerated reactor. Previous non-aerated experiments with *Zymomonas mobilis* have given kinetic parameters in the same range, except for specific ethanol productivity, which was three times higher than our values [15].

Mannitol as substrate

Since oxygen may be a crucial factor in metabolism of mannitol [14], some preliminary experiments were carried out under different oxygen regimes. One open and five sealed bottles, containing different liquid volumes, were incubated in a shaker for 67.5 h and end concentrations of oxygen were measured (Figure 2). The results demonstrated a clear dependency on air access: the activity in the sealed bottles increased with decreasing medium volumes, and in the open bottle and the one with lowest liquid volume, all

Table 1 Operating constants and kinetic parameters

Figure	1A	1B	4A	4B	5A	5B	6 (big)	6 (small)
Initial substrate concentration (g L^{-1})	18 G synthetic	16 G synthetic	38 M synthetic	38 M synthetic	16 G 19 M synthetic	17 G 21 M synthetic	17 M extract	17 M extract
Air/N_2 (L L^{-1} h^{-1})	0.05 N	0.20 A	0.20 A	0.20 A	0.20 A	0.05 N/0.10 A		
OTR (mmol O_2 L^{-1} h^{-1})	0	3.56	4.75	6.19[a]	4.19	0/2.91		
Max. CER (mmol CO_2 L^{-1} h^{-1})	8.68	20.10	4.26	19.91	17.30	8.04		
(h after inoculation)	(14.7)	(13.8)	(10.5)	(6.7)	(10.5)	(19.0)		
Specific growth rate μ (h^{-1})	0.36	0.63	0.63	0.71	0.70	0.34/0.05[c]		
Specific ethanol productivity q_p (g g^{-1} h^{-1})	0.74	0.77	0.73	0.04	1.28	0.74/0.59[c]	0.02	0.03
dS/dt (g L^{-1} h^{-1})	0.45	0.96	0.45	0.98	1.03/0.82[b]	0.32/0.85[d]	0.61	1.06
dP/dt (g L^{-1} h^{-1})	0.18	0.29	0.17	0.05	0.39/0.32[b]	0.14/0.28[d]	0.37	0.56
Ethanol yield Y_{PS} (g g^{-1})	0.39	0.31	0.38	0.05	0.37/0.39[b]	0.44/0.33[d]	0.61	0.53
Biomass yield Y_{XS} (g g^{-1})	0.06	0.13	0.10	0.08	0.10	0.08/0.02		

dS/dt, dP/dt and Y_{PS} were calculated from end concentrations or, when substrate was totally consumed, the last sample before substrate exhaustion. μ and q_p were calculated for the initial exponential phase of growth and represent maximum values. For the calculation of q_P, biomass was estimated as the average of two succeeding samples. Y_{XS} was calculated for the growth period.
M = mannitol, G = glucose, A = air, N = nitrogen, OTR = oxygen transfer rate, CER = CO_2 emission rate, dS = substrate consumption, dP = ethanol production.
[a]Dissolved oxygen maintained at 25%. The value represents the maximum OTR, which coincided with the maximum CER.
[b]Calculated for the periods 0–18 h and 18–32 h. Glucose was exhausted at 18 h.
[c]Calculated for the initial growth and the renewed growth initiated after aeration.
[d]Calculated for the periods 0–23 h and 47.5–75 h.

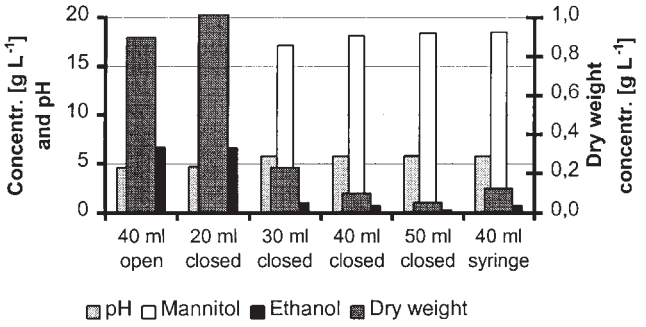

Figure 2 Mannitol metabolism: end concentrations in six different shake-bottle experiments after 67.5 h of incubation: 40 ml medium with loose screw cap, 20, 30, 40, and 50 ml medium in bottles with sealed screw caps, and 40 ml medium sealed with a sceptre penetrated with a syringe to take up pressure increase. The total bottle volume was 70 ml.

the mannitol was consumed. Both bottles had high ethanol and biomass concentrations, a yield of 0.36 g ethanol (g mannitol)$^{-1}$ and a pH of 4.6. In all the other bottles the activity was very low, with almost no mannitol consumption and a pH of 5.8. The bottle with a syringe to allow for expansion volume showed a 3-ml volume increase due to pressure rise, without affecting growth. When the sealed bottles were opened, growth was observed (results not shown).

Mannitol fermentation was further investigated in six reactors (Figure 3). The data confirmed the bottle experiments: no growth or mannitol consumption was observed in the two anaerobic reactors, and they were stopped after 42 h. Increasing OTR increased both growth rates and Y_{XS} in the aerated reactors. Within the total fermentation period of 140 h, the three aerated reactors reached a maximum dry weight concentration: 1.0 g L^{-1} after 24 h, 0.8 g L^{-1} after 67 h, and 0.6 g L^{-1} after 90 h. These maximum values coincided with the exhaustion of mannitol. In the open non-aerated reactor, cell mass was still increasing when the

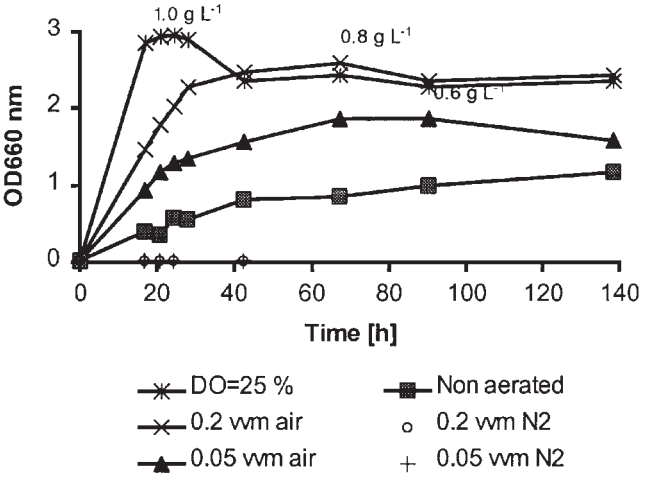

Figure 3 Mannitol metabolism: optical density in six different fermentor experiments: dissolved oxygen concentration maintained at 25%, 0.2 and 0.05 L air (L medium min)$^{-1}$ (OTR = 3.50 and 1.84 mmol O_2 L^{-1} h^{-1}), no aeration, and 0.2 and 0.05 L N_2 (L medium min)$^{-1}$. All reactors contained the same medium with initial mannitol concentration of 20 g L^{-1}. The numbers give the maximum dry weight concentrations for the three aerated cultures. vvm = L (L medium min)$^{-1}$.

experiment was stopped. However, growth was, as in the glucose experiments, clearly inhibited before exhaustion of mannitol. The dissolved oxygen concentration in the non-DO-regulated reactors quickly dropped to zero after inoculation, resulting in oxygen-limiting conditions. In the two lowest-aerated reactors, oxygen concentration started to rise again about 40 h after mannitol depletion, while in the third aerated reactor the oxygen started to rise above 25% 16 h after substrate depletion (results not shown). This demonstrates that the cells still could maintain high metabolic activity after the substrate was exhausted, and at least some of this activity can be attributed to production of organic acids from ethanol. The ethanol concentration was generally observed to decrease after substrate depletion. The total ethanol yield for the oxygen-limited reactors was in the range 0.31–0.35 g ethanol (g mannitol)$^{-1}$, while it was 0.12 g ethanol (g mannitol)$^{-1}$ in the 25% DO reactor. In this fermentation accumulation of organic acids was observed.

The inability of mannitol to support strictly anaerobic growth is probably caused by a deregulation of the redox balance [14]. Anaerobic growth on glucose is balanced because the 2 NADH produced in the bacterial Entner–Doudoroff pathway are consumed when ethanol is produced from pyruvate, thereby regenerating NAD^+. Mannitol probably enters this pathway via fructose produced by mannitol dehydrogenase, an enzyme that also converts NAD^+ to NADH. The overall result is an excess production and accumulation of NADH under anaerobic conditions, since regeneration of NAD^+ presumably does not take place. The reason may be a lack of transhydrogenase, which converts NADH to NADPH, although general textbooks claim that procaryotes possess this enzyme [4,6]. This would lead to a compartmentation of the redox couples NAD^+/NADH and $NADP^+$/NADPH. NADH is predominantly a catabolic reducing equivalent, whereas NADPH is mainly involved in anabolic processes [18].

A fermentation with an initial mannitol concentration of 38 g L^{-1} is presented in Figure 4a. Dissolved oxygen quickly dropped below the detection limit, and a maximum CER of 4.3 mmol CO_2 L^{-1} h^{-1} was reached at 10.5 h, corresponding to one-fifth of the maximum rate observed when the organism was grown on glucose (Figure 1b, Table 1). However, the CER was maintained at this level throughout the experiment. The growth rate was very low from 20 h and onwards, in spite of the remaining mannitol in the medium. From Figure 4a it is readily seen that ethanol production was not directly growth-associated, giving similar ethanol yields during both growth and stationary phases. The total ethanol yield of 0.38 g ethanol (g mannitol)$^{-1}$ was the maximum achieved in mannitol medium (Table 1). Carbon balance at 70 h including biomass, CO_2, ethanol and acetate accounted for 81% of the carbon consumed.

This mismatch in the balance could be due to evaporation of ethanol, which is a problem in ethanol fermentations. Carbon balances earlier in the fermentation period accounted for more of the carbon. Concerning organic acids, only traces of acetate were identified by HPLC. Thus, with the carbon source present in the medium and under oxygen limiting conditions, there seemed to be almost no production of organic acids. The values of q_P, dS/dt, dP/dt and Y_{PS} were similar to those of the anaerobic fermentation

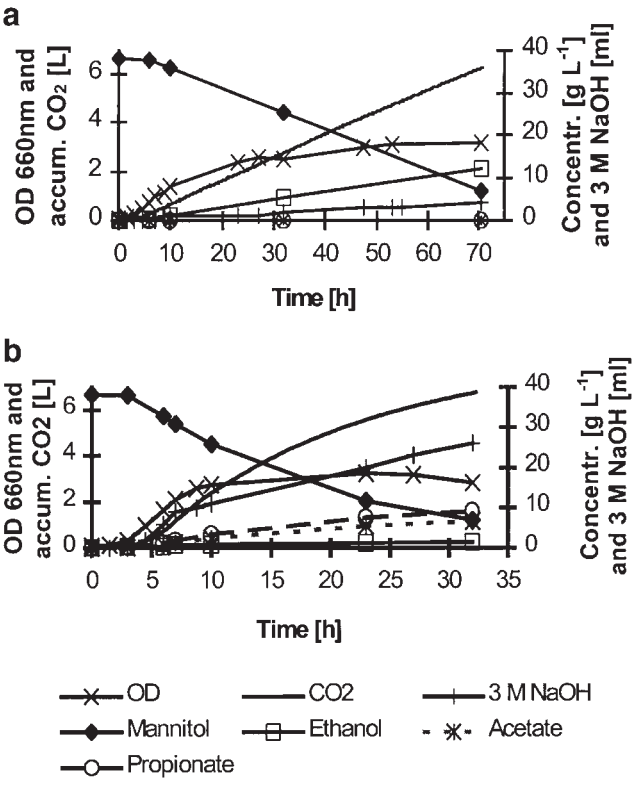

Figure 4 Mannitol metabolism: optical density, accumulated CO_2, consumption of 3 M NaOH, mannitol, ethanol, acetate and propionate concentrations in (a) fermentor aerated with 0.20 L air (L medium min)$^{-1}$ (OTR = 152 mg O_2 L^{-1} h^{-1}) and (b) aerated fermentor maintained at 25% DO.

on glucose. μ was the same as in the aerated glucose fermentation, while Y_{XS} was in the range between the anaerobic and aerated glucose fermentations (Table 1).

Another fermentation in 38 g L^{-1} mannitol medium is shown in Figure 4b. The oxygen concentration was maintained at 25% DO and the maximum CER was 19.91 mmol CO_2 L^{-1} h^{-1}, similar to the maximum achieved in the aerated glucose fermentor (Table 1). From this peak value, however, the CER decreased steadily until the experiment was ended at 32 h. At this point there was still mannitol in the medium, only traces of ethanol and a biomass concentration of 1.0 g L^{-1}. The high consumption of NaOH was probably associated with the pronounced production of acetate and propionate, which yielded end concentrations of 6.7 and 9.2 g L^{-1}, respectively. At 10 h there was a clear halt in growth, at a propionate concentration of 3.5 g L^{-1}. The tendency for the period of 10–32 h was nearly a complete halt in growth, accumulation of organic acids and a gradual decrease in activity (Figure 4b). This pattern was contrasted by the oxygen-limited experiment of Figure 4a, where only traces of organic acids were found after 70 h, and the activity was constant for the whole period. This suggested a first phase of inhibition of growth, followed by a gradual inhibition of activity as the concentration of organic acids increased. Small organic molecules have inhibitory effects on microorganisms, the most common being lactic, acetic and propionic acids [1]. It has also been reported that mixtures of organic acids reduce growth rate more than either acid alone, suggesting a synergetic effect [16]. Although it is often assumed that the undissociated molecule is the toxic form of weak acids, giving stronger inhibition at low pH, dissociated acids also inhibit microbial growth [3]. The acid production in our high-DO fementations suggests that under aerobic conditions the bacteria followed a different fermentation pattern than under oxygen-limiting conditions. The unusual accumulation of organic acids under aerobic conditions may indicate that the bacteria possess a limited respiratory metabolism. Mannitol fermentations with DO of 35% yielded even higher concentrations of organic acids, while no ethanol was detected (results not shown). The molar propionate/acetate ratio increased with the oxygen concentration, from 1.1 at 25% DO to 6–7 at 35% DO. Propionibacteria also show a high production of acetate and propionate [9].

In all experiments cell dry weight concentration never exceeded 1.3 g L^{-1}, irrespective of initial substrate concentration and oxygen supply. This could be due to depletion of an essential nutrient in the medium. The only factor that is reported to be required for growth is nicotinic acid [11], but this factor should have been supplied by the yeast extract. However, the experiments suggest an anabolic growth factor, since only growth is halted, not activity. Cessation of growth could also indicate product inhibition. Okamoto *et al* reported [12] that an ethanol concentration of about 50 g L^{-1} inhibited growth, but this is far higher than our maximum concentrations. The halt in growth in our aerobic experiments at 25 and 35% DO was observed at different concentrations of acetic and propionic acid. Thus, accumulation of organic acids was probably not the cause of inhibition.

Ethanol yield was highly dependent on oxygen concentration (Table 1), because excess oxygen led to production of organic acids and thereby reduction of the yield. Thus the optimisation of ethanol yield will be a compromise between the need of oxygen for NADH oxidation and the minimisation of organic acid production. Exhaustion of the substrate should also be avoided, since this led to ethanol consumption and production of organic acids. The best procedure for optimisation of ethanol yield seems to be a start up with a low OTR, and then a gradual increase in OTR to about 4.75 mmol O_2 L^{-1} h^{-1} in the stationary phase. A further increase in OTR may also be possible as long as the culture is oxygen-limited. This would satisfy the growing oxygen demand, but at the same time avoid excessive production of organic acids.

Okamoto *et al* [11] reported that *Z. palmae* is a facultatively anaerobic bacterium, able to ferment a range of carbohydrates, including mannitol. The use of the term *fermentation* may be confusing, since it has a two-fold definition: (1) substrate metabolised without exogenous electron acceptor; (2) in industrial microbiology: *any* of a wide range of processes carried out by microorganisms, regardless of whether fermentative or respiratory metabolism is involved [17]. Our results confirmed an ability to ferment mannitol according to the second definition, but we were not able to grow *Z. palmae* on mannitol under purely anaerobic conditions.

Glucose and mannitol as mixed substrate

Concerning the fermentation of seaweed extract, a mixture of glucose and mannitol was used to test whether their metabolism would occur simultaneously or successively. In an aerated fermentation with mixed substrate, glucose was the preferred substrate, indicating catabolic repression of mannitol conversion (Figure 5a). The glucose was probably totally consumed after 18 h, indicated by a shift in CER. The maximum CER at 10.5 h coincided with growth limitation, but unlike aerated growth on glucose only (Figure 1b), CER was not stabilised at this maximum. The activity was much better with glucose still present, visualised in Figure 5a as a shift in the CO_2 curve at 18 h. The maximum cell density at 27 h (1.3 g L^{-1}) was the highest achieved in the entire series of experiments, and the total yield of 0.40 g ethanol (g glucose + mannitol)$^{-1}$ was also the highest for an aerated reactor (Table 1). The q_p was considerably larger than in the other experiments. This shows that the mixture of both laminaran and mannitol found in a seaweed extract might have a positive effect on the ethanol production. In such a case, however, laminaran has to be hydrolysed to glucose first. Carbon balance based on biomass, CO_2, ethanol, propionate and acetate accounted for 96% of the consumed carbon in glucose and mannitol.

An anaerobic fermentation of these mixed substrates is presented in Figure 5b. Based on the previous experiments, it was expected that glucose would be consumed and that mannitol would remain unaffected. In the first phase of the experiment both CO_2 development and growth were similar to that of the anaerobic culture on glucose (Figure 1a).

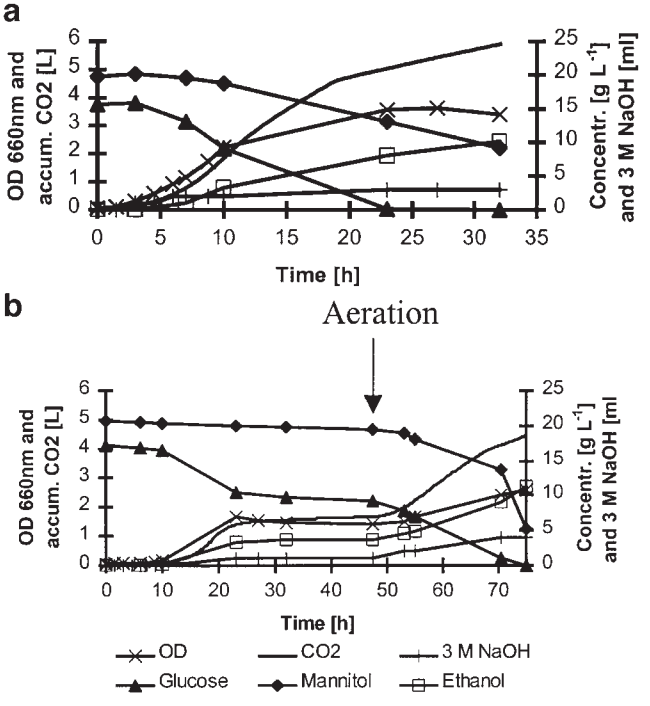

Figure 5 Mixed glucose and mannitol metabolism: optical density, accumulated CO_2, consumption of 3 M NaOH, glucose, mannitol, and ethanol concentrations in (a) fermentor aerated with 0.20 L air (L medium min)$^{-1}$, OTR = 134 mg O_2 L^{-1} h^{-1}, and (b) fermentor sparged with 0.05 L N_2 (L medium min)$^{-1}$. At 47.5 h the N_2 was changed to 0.10 L air (L medium min)$^{-1}$.

However, after initial growth and glucose consumption, growth totally ceased after 23 h, with 10.3 g L^{-1} glucose still remaining in the medium. The consumption of substrate also ceased, and in the following day only 1 g L^{-1} glucose and 0.5 g L^{-1} mannitol were consumed. The reason may be a disturbance of the redox balance, caused by the small consumption of mannitol. In all the other experiments, termination of growth was not associated with a loss of substrate consumption activity. In an attempt to regain activity, the reactor was aerated after 47.5 h. The cells started to grow again, but now both glucose and mannitol were consumed simultaneously, with a pronounced increase in mannitol consumption when all the glucose was gone. There was also an increasing mismatch in the carbon balance throughout this experiment, accounting for only 66% at 75 h. A strong mismatch in the carbon balance was observed in the experiments which ran for the longest time (Figures 1a, 4a and 5b), and could at least partly be caused by ethanol evaporation.

Seaweed extract

Extraction experiments in our laboratory with fresh autumn fronds of *L. hyperborea* at 20% dry weight yielded extracts with both mannitol and laminaran concentrations up to 30 g L^{-1}. Extracts made from dried material may give higher concentrations.

Z. palmae was also tested for activity in this seaweed extract. In order to test if the high salt content in seaweed [7] could inhibit *Z. palmae*, shake flask experiments were run with mannitol medium containing 0–3% NaCl. No inhibitory effect on growth was observed. Also, tests with pure extract and dilutions with tap water and medium showed that the activity of *Z. palmae* was not inhibited by the extract (results not shown).

Two identical shake flasks with 75 and 150 ml of seaweed extract were inoculated with the pellet of a preculture grown on synthetic mannitol medium. The bacteria were able to utilise the mannitol in the extract for ethanol production (Figure 6). Ethanol production and mannitol consumption were faster in the small volume, again demonstrating dependence on oxygen transfer. Ethanol yield was for the small volume 0.53 g ethanol (g mannitol)$^{-1}$ after 11.7 h, while the large volume culture had a yield of 0.61 g ethanol (g mannitol)$^{-1}$ after 21.9 h. These ethanol yields are

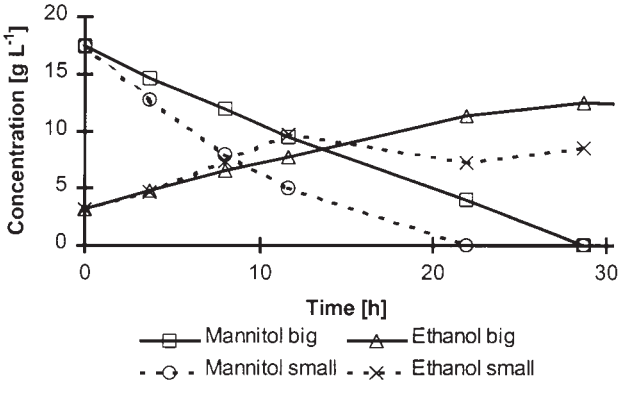

Figure 6 Metabolism of seaweed extract: mannitol and ethanol concentrations in two identical shake flasks with small (75 ml) and large working volumes (150 ml).

higher than the theoretical yield of 0.51 g g^{-1}, indicating that *Z. palmae* can utilise other carbon sources in the extract. Both d*S*/d*t* and d*P*/d*t* in the small volume were higher than in all the fermentor experiments (Table 1), indicating higher activity in more dense cultures. After the depletion of mannitol, a small increase in organic acids was observed. Analyses of the laminaran content showed no consumption, confirming the results of earlier shake-flask experiments.

Conclusions

Z. palmae metabolised glucose under strictly anaerobic conditions. Anaerobic growth on mannitol was not observed, probably due to a deregulation of the redox balance. Excessive aeration led to a change in the fermentation pattern, with pronounced production of acetate and propionate. These acids also accumulated when the substrate was depleted, concomitant with a consumption of ethanol. However, under oxygen-limiting conditions the bacteria readily produced ethanol in synthetic mannitol medium, yielding 0.38 g ethanol (g mannitol)$^{-1}$. *Z. palmae* was also successfully applied for fermentation of mannitol from *L. hyperborea* extracts.

Acknowledgements

This work was supported by the Norwegian Research Council (NFR) and Norsk Hydro ASA, Bygdøy allé 2, Oslo, Norway.

References

1 Blom H and C Mørtvedt. 1991. Anti-microbial substances produced by food associated micro-organisms. Biochem Soc Trans 19: 694–698.
2 Dobson SJ and PD Franzmann. 1996. Unification of the genera *Delaya* (Baumann *et al* 1983), *Halomonas* (Vreeland *et al* 1980), and *Halovibrio* (Fendrich 1988) and the species *Paracoccus halodenitrificans* (Robinson and Gibbons 1952) into a single genus, *Halomonas*, and placement of the genus *Zymobacter* in the family *Halomonadaceae*. Int J Syst Bact 2: 550–558.
3 Eklund T. 1983. The antimicrobial effect of dissociated and undissociated sorbic acid at different pH levels. J Appl Bacteriol 54: 383–389.
4 Gottschalk G. 1986. Bacterial Metabolism, 2nd edn. Springer-Verlag, NY.
5 Haug A and A Jensen. 1954. Seasonal variations in the chemical compositions of *Alaria esculenta*, *Laminaria saccharina*, *Laminaria hyperborea* and *Laminaria digitata* from northern Norway. Report No. 4, Norw Inst Seaweed Res, Department of Biotechnology, Trondheim, Norway.
6 Ingraham JL, O Maaløe and FC Neidhardt. 1983. Growth of the Bacterial Cell. Sinauer Associates Inc, Sunderland, MA, USA.
7 Jensen A and A Haug. 1956. Geographical and seasonal variation in the chemical composition of *Laminaria hyperborea* and *Laminaria digitata* from the Norwegian coast. Rep Norw Inst Seaweed Res No. 14, Department of Biotechnology, Trondheim, Norway.
8 Kim SY, JH Kim and DK OH. 1997. Improvement of xylitol production by controlling oxygen sypply in *Candida parapsilosis*. J Ferment Bioeng 83: 267–270.
9 Moat GA and JW Foster. 1995. Microbial Physiology, 3rd edn. Wiley-Liss, NY.
10 Myklestad S. 1978. β-1,3-Glucan in diatoms and brown seaweeds. In: Handbook of Psychological Methods. Physiological & Biochemical Methods (JA Hellebust and JS Craigie, eds), pp 133–135, Cambridge University Press, Cambridge.
11 Okamoto T, H Taguchi, K Nakamura, H Ikenaga, H Kuraishi and K Yamasato. 1993. *Zymobacter palmae* gen nov, sp nov, a new ethanol-fermenting peritrichous bacterium isolated from palm sap. Arch Microbiol 160: 333–337.
12 Okamoto T, H Taguchi, K Nakamura and H Ikenaga. 1994. Production of ethanol from maltose by *Zymobacter palmae* fermentation. Biosci Biotech Biochem 58: 1328–1329.
13 Percival E and RH McDowell. 1967. Chemistry and Enzymology of Marine Algal Polysaccharides. Academic Press, London.
14 Quain DE and CA Boulton. 1987. Growth and metabolism of mannitol by strains of *S. cerevisiae*. J Gen Microbiol 133: 1675–1684.
15 Rogers PL, KJ Lee and DE Tribe. 1979. Kinetics of alchohol production by *Zymomonas mobilis* at high sugar concentrations. Biotechnol Lett 4: 165–179.
16 Rubin HE. 1978. Toxicological model for a two-acid system. Appl Environ Microbiol 36: 623–624.
17 Singleton P and D Sainsbury (eds). 1993. Dictionary of Microbial and Molecular Biotechnology. John Wiley & Sons, Chichester.
18 Van Dijken JP and WA Scheffers. 1986. Redox balances in metabolism of sugar by yeasts. FEMS Microbiol Rev 32: 199–224.