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Effect of glucose, maltose, soluble starch, and CO_2 on the growth of the hyperthermophilic archaeon *Pyrococcus furiosus*

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Abstract The hyperthermophilic archaeon *Pyrococcus furiosus* was cultivated in batch and continuous fermentations on different carbon substrates. The cultivation of *P. furiosus* on soluble starch as the only carbon source resulted in cell densities three times higher than in cultivations on maltose, 1.06×10^{10} cells/ml compared to 3.4×10^9 cells/ml. The yield coefficient, $Y_{x/s} = 0.12$ g/g, and the growth rate, $\mu = 0.33\ h^{-1}$, were almost equal on soluble starch and on maltose, but on glucose no growth could be detected. An inhibitory effect of glucose, when added to other carbon substrates, also could not be found. Isobutyric and isovaleric acid were detected as novel metabolites produced by *P. furiosus*. Inhibitory effects of these acids, as well as of the well-known products acetic acid, propionic acid, and alanine, could be precluded. Concentrations of 10% CO_2 in the gas supply respective to the exhaust gas enhanced the growth of *P. furiosus* significantly. The maximum cell number was two orders of magnitude higher than was observed with pure nitrogen. Further increase of the CO_2 concentration up to 100% had no significant effect on the growth of *P. furiosus*.

Key words *Pyrococcus furiosus* · Fermentation · Starch · Maltose · Glucose · Carbon dioxide · High cell density · Inhibition · Volatile fatty acids · Alanine

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Introduction

The hyperthermophilic archaeon *Pyrococcus furiosus* grows at temperatures up to 103°C (Fiala and Stetter 1986). This species produces interesting enzymes such as extremely heat-stable amylases, pullulanases, and proteases (Antranikian 1990; Kengen et al. 1996; Klingenberg et al. 1991). Unique possibilities, such as bioreduction of carboxylic acids (van den Ban et al. 1999), make the use of *Pyrococcus furiosus* interesting for novel production systems. To gain enzymes or to use the organism directly in production systems, high cell densities are required. The cell densities recorded so far are mainly less than 3.0×10^9 cells/ml; however, Raven and Sharp (1997), Krahe (1998), and Krahe et al. (1996) have reported cell densities up to 3.0×10^{10} cells/ml. These results were achieved with maltose as the only carbon source. Even though the growth on different substrates was investigated, this was only done in vial experiments or under suboptimal conditions (Driskill et al. 1999; Raven and Sharp 1997; Fiala and Stetter 1986).

For the experiments presented in this article, the substrates glucose, maltose, and soluble starch were chosen to investigate the growth of *Pyrococcus furiosus*. The major aim was to increase the cell density by optimization of substrate supply and fermentation conditions. Different fermentation techniques, such as continuous, batch, and fed-batch cultivation in 2-l foil fermentors, were performed.

Besides the liquid medium, the gassing of the culture has an important influence on the cultivation of microorganisms (Rüdiger et al. 1992). It is recorded that *P. furiosus* grows better if supplied with a certain amount of carbon dioxide. The most common gas mixture is 80% N_2 and 20% CO_2 (Krahe 1998; Nakashimada et al. 1998; Rüdiger et al. 1992; van den Ban et al. 1999). Nevertheless, some investigators worked without CO_2 and reached cell densities that are equal to fermentations with CO_2 (Raven et al. 1992; Raven and Sharp 1997; Schäfer and Schönheit 1991, 1992). The archaeon *Pyrococcus furiosus* metabolizes sugars to mainly acetic acid and CO_2 (Fiala and Stetter 1986; Krahe et al. 1996; Krahe 1998; Schäfer and Schönheit 1991, 1992). Thus, it should not be necessary to supply the cultivation with CO_2.

To determine the effect of CO_2 on the growth of *P. furiosus*, the organism was cultivated with concentrations from 0% to 100% CO_2 in the gas supply. The experiments were made on a defined medium with maltose or soluble starch as the single carbon source.

Materials and methods

Chemicals and organism

All amino acids were obtained from Sigma Aldrich (Deisenhofen, Germany), NaCl was from Akzo Nobel (Hamburg, Germany), and Zulkowsky starch was from Fluka (Deisenhofen, Germany). All other chemicals were obtained from Merck (Hannover, Germany). *Pyrococcus furiosus* was obtained from the German Collection of Microorganisms and Cell Cultures (DSM 3638).

Medium

In all experiments, *P. furiosus* was grown on a defined medium (Krahe 1998), with the following composition (in g/l): NaCl, 30; $CaCl_2 \cdot H_2O$, 0.1; KH_2PO_4, 1.4; $(NH_4)_2SO_4$, 1.3; $MgSO_4 \cdot 7H_2O$, 2.5; $NaSeO_3$, 0.0012; $Na_2WO_4 \cdot 2H_2O$, 0.0033; $FeSO_4 \cdot 7H_2O$, 0.035; cysteine $HCl \cdot H_2O$, 0.5; Resazurin, 0.001; trace element solution (10 ml/l) (Belay et al. 1984); amino cocktail (50 ml/l) (Raven and Sharp 1997); vitamin cocktail (1 ml/l) (Raven and Sharp 1997); and carbon source.

Preculture

Precultures were made in 100-ml serum bottles containing 45 ml medium with $NaHCO_3$ (1 g/l) and maltose (6.3 g/l) as substrate. The bottles were flushed with N_2 before they were sealed with butyl rubber septa. The pH was adjusted to 6.8 with 2N NaOH. After inoculation, the bottles were incubated for 12 h at 90°C.

Fermentor and fermentation setup

Setup for batch and fed-batch cultivation

For the fermentations, a 2-l foil fermentor from Bioengineering (Wald, Switzerland) was used. The fermentor was equipped with temperature and pH control. For titration, a 2N NaOH solution was used. The fermentation broth was gassed with nitrogen/carbon dioxide mixtures, and the exhaust gas was cooled to minimize evaporation losses.

The fermentation parameters were T, 90°C; stirrer speed, 1,800 rpm; gas flow rate, 0.4 vvm N_2/CO_2 mixture; pH online, 6.4; fermentation volume, 1.5 l. Fed-batch was performed by feeding complete medium with high substrate concentrations or by adding pulverized soluble starch stepwise.

Continuous fermentation setup

Setup and parameters were, except for the dilution, identical with the setup for batch cultivation. Fresh medium was prepared in a 20-l bottle. During fermentation, the bottle was stirred constantly, heated, and gassed with nitrogen. The dilution rate was regulated by a pump for the fresh medium and controlled by a balance. The volume in the fermentor was leveled by pumping the effluent from the surface.

Steady state was assumed after four residence times. To determine if steady state had been reached, cell density was determined three times over a period of 2 h. Samples and values were taken only if the cell density was constant; otherwise, the control procedure was repeated after two more residence times.

Measurement of redox value

The redox value was measured with a platinum redox probe (D1107A-PBA; Broadley and James, Santa Ana, CA, USA).

Determination of cell density

Cells were counted directly with a Neubauer chamber (depth, 0.02 mm) under a phase-contrast microscope. The optical density was measured at 600 nm between 0 and 0.4 with appropriate dilution.

Determination of $Y_{x/s}$

For the determination of $Y_{x/s}$, samples of the fermentation were taken immediately after inoculation and after reaching maximum cell number. The substrate concentration was measured by the method of Dubois et al. (1956). The cell dry weight was estimated by a cell weight published by Krahe (1998). At maximum cell number on maltose fermentations, the cell weight of *P. furiosus* was 1.0×10^{-13} g/cell. The calculation of $Y_{x/s}$ includes an uncertainty of 10%.

Analysis of alanine

Alanine was detected by high pressure liquid chromatography (HPLC) analysis. The HPLC column was an Ultrasphere ODS 5 µ (Beckman, Unterschleißheim, Germany) with diameter of 4.6 mm and length of 250 mm. The detector was a fluorescence HPLC-monitor RF 1002 (Gynotek, Unterschleißheim, Germany).

Analysis of organic acids

Organic acids were measured by gas chromatography. Samples were mixed with metaphosphoric acid. A Widebore Nukul (Supelco, Deisenhofen, Germany) column with length of 15 m and an infrared detection system (0.53) were used.

Results and discussion

Batch and fed-batch fermentations on different carbon substrates

Glucose (initial concentration, 5 g/l), maltose (6.3 g/l), and soluble starch (15 g/l) were chosen as substrates to examine the fermentation behavior of *Pyrococcus furiosus*. The cultures were gassed with 0.4 vvm Biogon (20% CO_2, 80% N_2).

Growth on glucose could not be detected in the batch experiments. Driskill et al. (1999) has reported low growth of *P. furiosus* on glucose, but only in vial experiments. Growth curves on maltose and soluble starch are shown in Fig. 1. On soluble starch, the lag phase was longer. For all experiments, maltose was used as the only carbon source in the precultures, and therefore a substrate adaptation had to take place. The maximum growth rates were almost equal on maltose ($\mu_{max,malt} = 0.33\ h^{-1}$) and on soluble starch ($\mu_{max,sol\ starch} = 0.31\ h^{-1}$). A large difference occurred in maximum cell density and in growth behavior. The growth on maltose stopped at a cell number of 3.4×10^9 cells/ml whereas the growth on soluble starch resulted in a cell number of 1.06×10^{10} cells/ml, three times greater than on maltose. After reaching the maximum, on maltose the cell number decreased slowly, but on soluble starch it declined rapidly. The values of the cell density on maltose and the growth rate, $\mu_{max,malt}$, are similar overall to values reported by Krahe (1998), Schäfer and Schönheit (1992), and Schicho et al. (1993). Cultivation of *P. furiosus* on soluble starch was reported by Fiala and Stetter (1986), Kengen et al. (1996), and Raven and Sharp (1997), but no growth curves were shown. Raven and Sharp (1997) reported a maximum cell number of 1.0×10^{10} cells/ml, equal to the results reported here, but gave no value for the maximum growth rate.

Starch consists of two fractions, amylose and amylopectin. To study the growth of *P. furiosus* on either of the two fractions, amylose (20 g/l) and waxy cornstarch (100% amylopectin, with only minor impurities of amylose, 10 g/l) were used as the single carbon source. On amylose, the growth rate was $\mu_{max,amylose} = 0.37\ h^{-1}$ and the maximum cell number was 8.0×10^9 cells/ml (Fig. 1). After reaching the maximum, cell density decreased rapidly. On amylopectin, the lag phase was long and growth was slow ($\mu_{max,amylopectin} = 0.20\ h^{-1}$). The maximum cell number was 2.1×10^9 cells/ml and was even lower than on maltose. Because of the poor solubility of the amylopectin and thereby the presence of many particles in the fermentation broth, the optical density could not be measured. Fermentation on amylose or amylopectin as the only carbon source has not been reported so far.

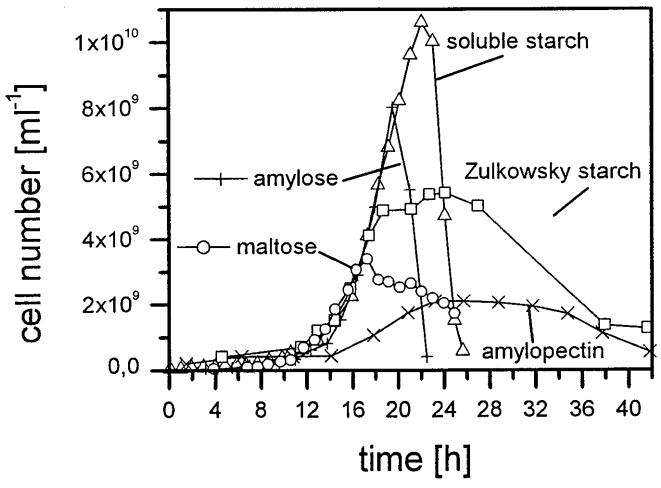

Fig. 1. Growth curves of batch fermentations on maltose (*circles*), soluble starch (*triangles*), amylose (*line with crossbars*), amylopectin (*crosses*), and Zulkowsky starch (*squares*)

Although the substrates were not fully consumed, the results of the experiments with maltose and soluble starch indicated substrate limitation by low solubility or transport limitations. To exclude substrate limitation, fed-batch experiments were carried out. The substrates were added continuously as a sterile, oxygen-free solution or in pulverized form in appropriate time steps. No effect of the feeding compared to simple batch experiments could be detected in fermentation on maltose. In fed-batch fermentations with soluble starch, the maximum cell number (8.5×10^9 cells/ml) was even lower than in batch experiments (1.06×10^{10} cells/ml). No explanation could be found.

In addition to the fed-batch fermentations, batch experiments with a more soluble starch were made. The special starch was treated by a method invented by Zulkowsky (1880) to decrease the size of the molecules and to increase the solubility. After the treatment, as much as 100 g/l of the Zulkowsky starch is completely soluble in water, compared to a maximum of 20 g/l of normal soluble starch; 40 g/l of this special starch was completely dissolved in the fermentation broth. The growth curve on Zulkowsky starch is shown in Fig. 1. The maximum growth rate, $\mu_{max,Zulkowsky} = 0.32\ h^{-1}$, was equal to that on soluble starch, but the maximum cell number of 5.4×10^9 cells/ml reached only half the number on normal soluble starch. This lower maximum cell number was, however, constant for more than 10 h. So far, a limitation (15 g/l of the substrate remained at the end of the fermentation) as well as an inhibition ($\mu_{max,Zulkowsky} = \mu_{max,sol\ starch} = \mu_{max,malt}$) by this substrate could not be detected. No experiments have been reported with Zulkowsky starch so far.

An overview of relevant data on the physiology of growth of *P. furiosus* on different carbon sources is given in Table 1. To control the absence of oxygen, the redox potential of the fermentation broth was measured on-line during all fermentations. Although the pH was constant (on-line and off-line measurements) and oxygen influx was avoided [permanent gassing with N_2 (80%)/CO_2 (20%), control by resazurin], the redox value of the fermentation broth increased over the fermentation period from –380 mV to –300 mV and decreased again to –400 mV in fermentations on soluble starch (Fig. 2). A fermentation broth that was not inoculated stayed constant at –380 mV for more than 36 h. Control of the redox value by adding cysteine or Na_2S did not enhance the growth either on maltose or on soluble starch.

The redox potential in fermentations of *P. furiosus* has been of no interest until the present time. Recently,

Table 1. Maximum cell number, maximum growth rate (μ), and the yield coefficient ($Y_{x/s}$) of *Pyrococcus furiosus* cultivated in batch fermentations on different carbon substrates

Substrate	Cell number (10^9/ml)	μ (h^{-1})	$Y_{x/s}$ (g/g)
Soluble starch	10.6	0.31	0.12
Amylose	8	0.37	0.13
Amylopectin	2.1	0.20	0.11
Zulkowsky starch	5.4	0.32	0.13
Maltose	3.4	0.33	0.12

Gassing was at 0.4 vvm Biogon (20% CO_2, 80% N_2)

reported unique bioreduction possibilities (van den Ban 1999) and the redox curves shown in this article make it interesting to take a closer look at the redox potential during the fermentation period. In aerated cultures, control of the fermentation with the redox potential was reported by du Preez et al. (1988). Kjaergaard (1977) reported also that the redox potential could be used as an indicator for microbial activity and that it would be an important factor in killing microorganisms. The results presented in this paper led to the conclusion that the redox potential could be used to control anaerobic conditions but not to control the fermentation and microbial activity of *P. furiosus*. The organism does not react obviously to changes in the redox potential between –400 mV and –300 mV.

To find inhibiting products, probes of the supernatant of fermentations on maltose and soluble starch were analyzed on organic acids and alanine (Fig. 3). In addition to the often-reported alanine, acetic acid, and propionic acid, two novel products were found, i.e., isobutyric and isovaleric acid. A possible inhibition of those acids was tested in fermentations with an initial concentration of 300 mg/l of either acid; 300 mg/l is equivalent to six- or fivefold, respectively, the concentration of the acids at the end of a batch fermentation on soluble starch. Cell number and growth rate equaled fermentations without the addition of the novel products found; inhibition by these products is unlikely, therefore.

An inhibition by acetic acid and propionic acid was described by Nakashimada et al. (1998) and denied by Krahe et al. (1996). In the experiments described here, inhibition by acetic acid or propionic acid could not be detected and is not responsible for the differences in the maximum cell densities on maltose and soluble starch.

An influence of alanine in concentrations up to 10 mmol/l on the growth of *P. furiosus* also could not be found. The ratio between the production of alanine and the production of acetic acid is 0.06 on maltose (1–16.67 mmol/l) as on soluble starch (5.5–0.92 mmol/l).

Continuous fermentation with glucose, maltose, and soluble starch

Continuous fermentations were carried out with a gassing rate of 0.4 vvm Biogon (20% CO_2, 80% N_2). Substrate concentrations were 5 g/l glucose, 6.3 g/l maltose, and 10 and

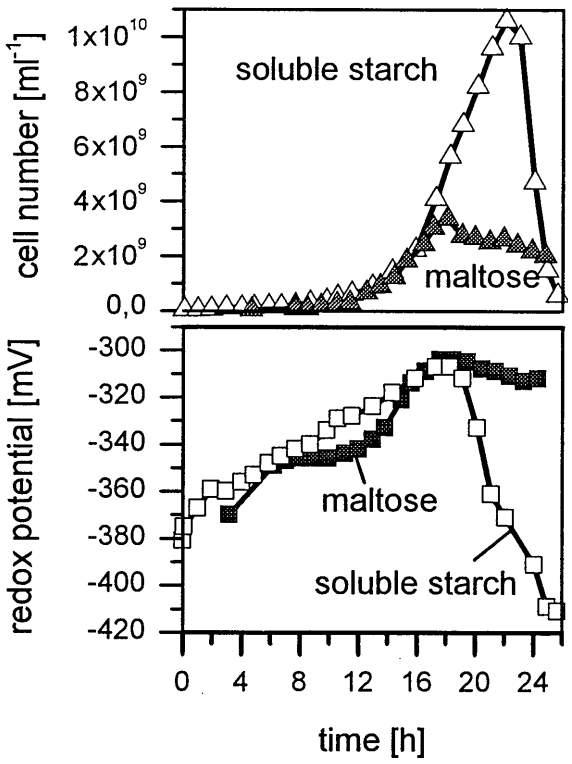

Fig. 2. Growth curves (*top*) and the corresponding redox potential (*bottom*) in batch fermentations on maltose (*closed symbols*) and soluble starch (*open symbols*)

15 g/l soluble starch. The dilution rate was varied, from D = 0.1 h^{-1} to D = 0.4 h^{-1}. Maximum cell numbers could be reached between D = 0.2 h^{-1} and D = 0.3 h^{-1}: on glucose, 2.5×10^7 cells/ml; on maltose, 2.0×10^9 cells/ml; and on soluble starch, 6.0×10^9 cells/ml (10 and 15 g/l). In washout experiments, values of μ_{max} for growth on maltose ($\mu_{max,malt}$, 0.42 h^{-1}) and soluble starch ($\mu_{max,sol\ starch}$, 0.41 h^{-1}) were determined (Fig. 4). Values for $Y_{x/s}$ (determined at D = 0.2 h^{-1}) were exactly the same as in the batch experiments (Table 2). $Y_{x,glucose}$ was determined to 0.02 g/g.

Glucose neither served sufficiently as the carbon substrate nor inhibited growth. Added amounts of glucose up to 10 g/l glucose to continuous fermentations on maltose or soluble starch did not have any detectable effect on cell density.

Fermentations on maltose and soluble starch with different concentrations of CO_2 in the gas supply

In most fermentation experiments so far, CO_2 has been used as part of the gas supply (Krahe et al. 1996; Nakashimada et al. 1998; van den Ban et al. 1999). Raven and Sharp (1997), however, reported fermentations without the use of CO_2 and reached cell densities equal to those reported by Krahe et al. (1996).

Continuous fermentations were carried out with maltose and soluble starch as the single carbon source. The concentration of CO_2 in the gas supply of the fermentations was

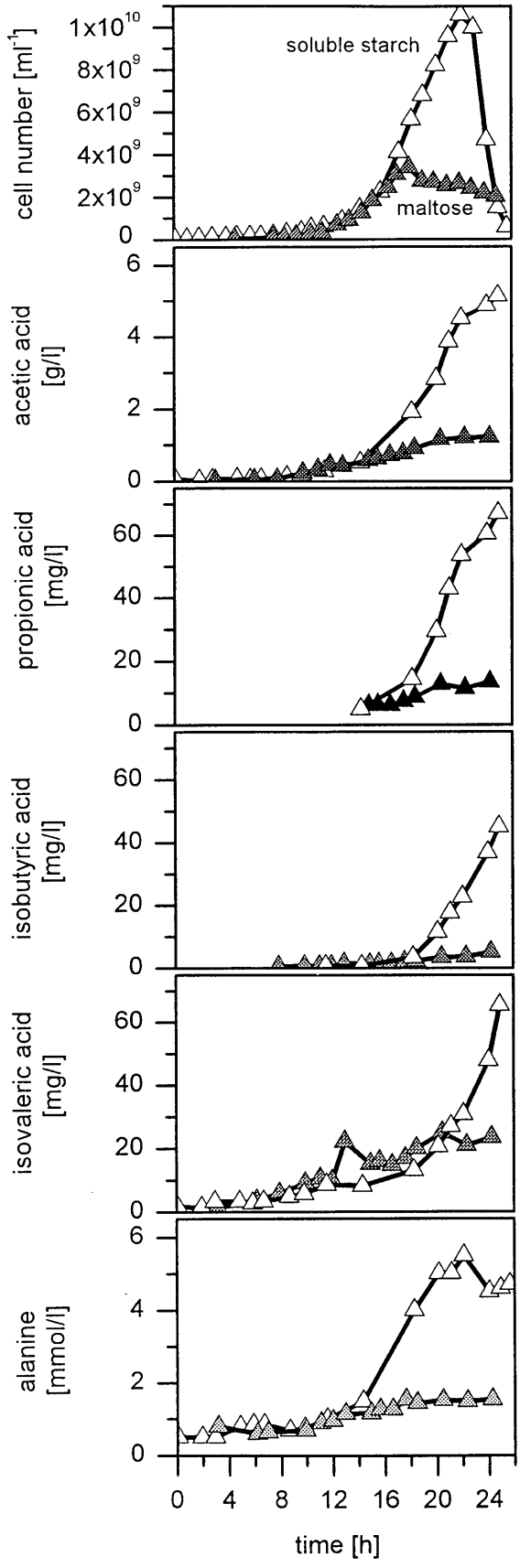

Fig. 3. Growth curves and the corresponding concentrations of detected organic acids and alanine in batch fermentations on maltose (*solid triangles*) and soluble starch (*open triangles*)

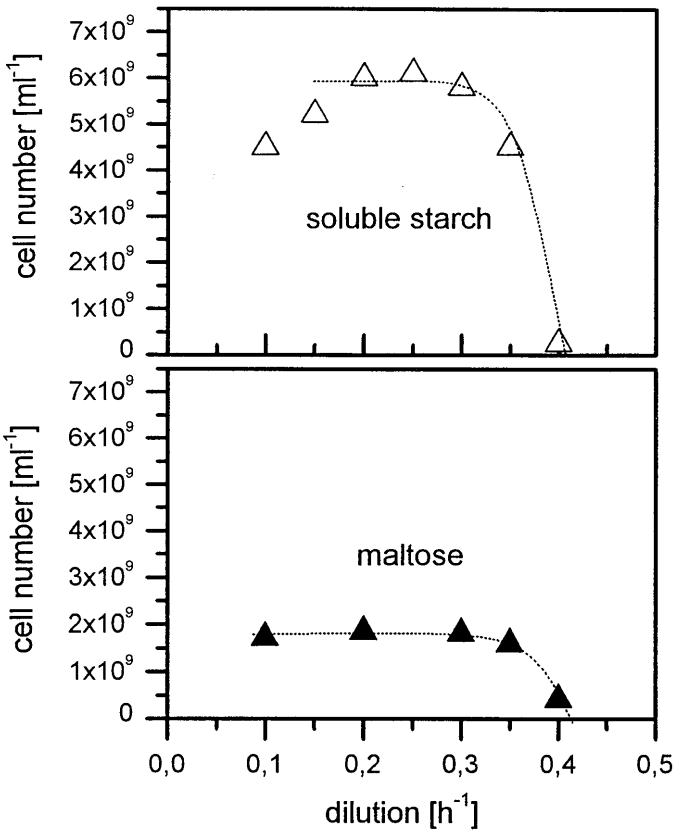

Fig. 4. Cell number at steady state relative to the dilution rate. The culture was gassed with 0.4 vvm Biogon (20% CO_2, 80% N_2). *Open triangles*, soluble starch; *solid triangles*, maltose

Table 2. Maximum cell number, maximum growth rate μ, and the yield coefficient $Y_{x/s}$ of *Pyrococcus furiosus* cultivated in continuous fermentations on maltose and soluble starch

Substrate	Cell number (10^9/ml)	μ (h^{-1})	$Y_{x/s}$ (g/g)
Soluble starch	6.0	0.41	0.12
Maltose	2.0	0.42	0.12

Gassing was at 0.4 vvm Biogon (20% CO_2, 80% N_2)

varied from 0% to 100% CO_2. Nitrogen was used as the inert gas. The concentration of CO_2 in the exhaust gas almost equaled the concentration in the gas supply because of the high gassing rate, 0.4 vvm (Table 3). Above 10% and up to 100%, no significant difference in cell density could be detected if *P. furiosus* was grown on soluble starch. On maltose, cell density increased slightly with increasing CO_2 concentrations between 10% and 100%. Under 10%, however, cell densities declined rapidly. If the cultures were gassed with pure nitrogen, which reduced CO_2 concentration in the exhaust gas below 1%, the cell densities were two magnitudes lower than in cultures gassed with at least 10% CO_2. The cultures were gassed more than 2 days with pure nitrogen, and no recovery of the cell density took place until the cultures were again gassed with CO_2. The results of the continuous fermentations could be confirmed by batch experiments.

Table 3. Cell densities in continuous fermentations with maltose or soluble starch as the only carbon source, depending on the CO_2 concentration in the gas supply

CO_2(%)	Substrate	
	Maltose	Soluble starch
0	0.018	0.064
2.5	–	0.315
10	1.9	6.1
20	2	6
30	1.7	–
40	1.8	–
50	1.8	6
100	2.1	6.1

The gassing rate was kept constant at 0.4 vvm; D=0.2 h^{-1}

Because *P. furiosus* metabolizes sugars to acetic acid and CO_2, a supply with CO_2 seems illogical, and it would probably possible to reduce the concentration of CO_2 in the supply gas. During two continuous fermentations, the gas supply stopped for more than 24 h for technical reasons. The cell densities stayed constant over this period, which indicates that a supply of CO_2 is not needed so long as the concentration of CO_2 in the exhaust gas is higher than 10%. An adaptation to CO_2 by the *P. furiosus* strain used here is unlikely, because a new culture from the DSMZ was used for the experiments to avoid such an adaptation.

Conclusion

It can be stated that *P. furiosus* grows not faster but to higher cell densities on soluble starch compared to maltose. Independent of the fermentation mode and the CO_2 concentration in the gas supply, the maximum cell number on soluble starch was three times higher than on maltose. It could also be shown that *P. furiosus* is able to grow on both fractions of starch, amylose and amylopectin. The use of a more soluble starch, treated by the method of Zulkowsky (1880), had no effect on the growth rate but had a negative effect on the maximum cell number. No explanation could be found.

In opposition to results published by Nakashimada et al. (1998), inhibition by acetic or propionic acid could not be found, which is in agreement with earlier studies of our group (Krahe et al. 1996). In addition to acetic and propionic acid, two novel metabolic products of *P. furiosus* were detected, i.e., isobutyric and isovaleric acid. Inhibition by these acids could not be found, nor could any effect be detected of a change in the redox potential between –400 mV and –300 mV on the growth of *P. furiosus*.

It could be shown that 10% CO_2 in the gas supply or the exhaust gas enhances the growth of *P. furiosus* significantly. The cell numbers were two orders of magnitude higher than in fermentations flushed with pure nitrogen, which led to CO_2 concentrations in the exhaust gas below 1%. An increase of the CO_2 concentration above 10% up to 100% did not have any significant effect on the cell number independent from the carbon source used.

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