J Ind Microbiol Biotechnol (2007) 34:17–25
DOI 10.1007/s10295-006-0157-5

ORIGINAL PAPER

Growth kinetics and Pho84 phosphate transporter activity of *Saccharomyces cerevisiae* under phosphate-limited conditions

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Received: 20 February 2006 / Accepted: 26 June 2006 / Published online: 16 November 2006

Abstract The effect of phosphate (P_i) concentration on the growth behavior of *Saccharomyces cerevisiae* strain CEN.PK113-5D in phosphate-limited batch and chemostat cultures was studied. The range of dilution rates used in the present study was 0.08–0.45 h^{-1}. The batch growth of yeast cells followed Monod relationship, but growth of the cells in phosphate-limited chemostat showed change in growth kinetics with increasing dilution rates. The difference in growth kinetics of the yeast cells in phosphate-limited chemostat for dilution rates below and above approximately 0.2 h^{-1} has been discussed in terms of the batch growth kinetic data and the change in the metabolic activity of the yeast cells. Immunological detection of a C-terminally *myc* epitope-tagged Pho84 fusion protein indicated derepressive expression of the Pho84 high-affinity P_i transporter in the entire range of dilution rates employed in this study. Phosphate transport activity mediated by Pho84 transporter was highest at very low dilution rates, i.e. 0.08–0.1 h^{-1}, corresponding to conditions in which the amount of synthesized Pho84 was at its maximum.

Keywords Phosphate · Pho84 transporter · Growth kinetics · *Saccharomyces cerevisiae*

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Introduction

The metabolism of *Saccharomyces cerevisiae*, a Crabtree positive yeast, is under influence of glucose and dissolved oxygen concentrations. Physiological behavior of *S. cerevisiae* in aerobic and anoxic chemostat culture under glucose limitation has been the subject of several studies. The metabolism of this yeast in aerobic glucose-limited chemostat culture is respiratory at low dilution rates and respiro-fermentative at higher dilution rates, the onset of fermentative metabolism occurs at critical dilution rate in which the respiratory capacity saturates [12]. Anoxic culture of *S. cerevisiae* is fermentative even at low dilution rates [9].

However, proper performance of the yeast cells is also highly dependent on phosphorous assimilation with involvement of the other bio-elements [16, 36]. Kinetics of growth of *S. cerevisiae* on substrates such as glucose, which are metabolized almost immediately after their uptake into the cells, is expected to be somewhat different from so-called ''conservative substrates'' such as phosphate (P_i), inorganic nitrogen and iron. These are substrates, which are not irreversibly consumed after uptake, but are stored within the cells [22]. Polymerization of P_i leads to insoluble polyphosphates formation, a P_i reserve content that has capacity to be used up by the cells under cells starvation conditions [5, 26].

Previous studies have indicated deviation of aerobic and anoxic growth of *S. cerevisiae* from Monod kinetics. In some cases, a shift from respiratory to respiro-fermentative metabolism has been reported as a reason for this phenomenon [7]. Also, deviation from Monod kinetics is related to cell requirements for growth factors under conditions of high growth rate and/or a respirofermentative metabolism [8].

Growth behavior of the *S. cerevisiae* cells in nutrient-limited batch and continuous cultures has been studied rather extensively [3, 6, 17, 21, 24, 29, 31, 35]. Although, there are few reports available on P_i-limited growth of the yeast cells, the growth kinetics of some microorganisms studied under P_i-limited chemostat culture show existence of meaningful correlation with either the internal or/and external P_i concentration. Working with bacterial and algal cells in some cases showed presence of a linear relationship between growth rate and intracellular phosphate concentration although a hyperbolic relationship has been obtained in some other cases [22]. Toda and Yabe [32] related the kinetics of batch growth of *S. carlsbergensis* under varying degrees of P_i limitation to the concentration of intracellularly accumulated P_i. It was found that the uptake rate of inorganic phosphate into the cells was regulated to maintain its intracellular concentration constant as long as P_i was available in the extracellular medium. On the other hand, the growth rate of the small marine yeast *Rhodotorula rubra* in continuous culture was related to the external P_i concentrations according to the Monod equation [4, 31].

Regulation of cellular reactions in response to external nutrient levels is fundamental to all living cells. To achieve this, the cells make use of numerous and varied mechanisms by which changes in the extracellular conditions can be sensed. P_i transport across the plasma membrane of *S. cerevisiae* is mediated by several specific plasma membrane transport systems, low- and high-affinity systems [26]. When the cells sense a P_i limitation in the growth media, there is an increased production of a high-affinity transport system and of secreted phosphatases that scavenge P_i from a variety of organic compounds through the action of periplasmically located acid phosphatases and alkaline phosphatases, involved in releasing P_i from intracellular substrates [23]. Activities and mechanisms of the cellular P_i uptake by *S. cerevisiae* have been thoroughly reviewed elsewhere [26].

In *S. cerevisiae* the active transport of P_i as well as many other P_i starvation responses, is regulated by the P_i signal transduction pathway (*PHO* pathway) [2].

The high-affinity system consists of two P_i transporters, Pho84 and Pho89, the gene products of *PHO84* and *PHO89*. The expression of both *PHO84* and *PHO89* is strongly regulated by P_i concentration [2, 18]. Pho84 is responsible for the majority of P_i uptake into the cells while the contribution of Pho89 and the recently characterized Pho87, Pho90 and Pho91 transporters appear to be lower [13, 18, 38]. It has previously been shown that the Pho84-mediated P_i uptake into cells during mid- to late-exponential growth in low-P_i medium is favored at an external P_i concentration of 50–70 μM [19, 25]. In most of the previous work on the kinetics and mechanisms of phosphate transport in *S. cerevisiae*, shake flask (batch) cultivation has been employed. However, chemostat system offers a more precise cultivation condition [1, 20, 28, 37].

The objective of the present study was to investigate the kinetics of the growth of *S. cerevisiae* CEN.PK113-5D cells as a function of phosphate concentration in both batch and chemostat cultivation. The expression of the Pho84 transporter at different dilution rates in the chemostat system and the relationship between growth, physiological and phosphate transport characteristics of these cells when grown under chemostat cultivation conditions was also explored.

Materials and methods

Materials

D-glucose and ethanol enzymatic bio-analysis kits were obtained from Boehringer–Manneheim/R-Biopharm, Sweden. ^{32}[P]orthophosphate (carrier-free, 0.1 Ci/μM, 1 mCi = 37 MBq), horseradish peroxidase (HRP)-conjugated antimouse-Ig-antibody (from sheep) and enhanced chemiluminescence detection kit were obtained from Amersham Biosciences, UK. Anti-*myc* antibody was obtained from Invitrogen, the Netherlands. The other materials were reagent grade and obtained from commercial sources.

Yeast strain and growth medium

Saccharomyces cerevisiae strain CEN.PK113-5D/Pho84-C*myc* (*MAT*a *MAL2-8c SUC2 ura3-52*) has been used in this study [15]. Cells were grown on YPD agar plates (1% yeast extract, 2% peptone, 2% glucose and 2% agar) and maintained at 4°C.

The cells were aerobically cultivated in defined, synthetic mineral medium prepared essentially as described by Verduyn et al. [33].

The main elements were composed of: Glucose, 30 g l^{-1}; $(NH_4)_2SO_4$, 5 g l^{-1}; $MgSO_4{\cdot}7H_2O$, 0.5 g l^{-1}; KH_2PO_4, 0–1,000 μM and KCl, 21–22 mM (the final

concentration of potassium was 22 mM). Trace elements additions per liter were: EDTA, 15 mg; $ZnSO_4 \cdot 7H_2O$, 4.5 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; $CoCl_2 \cdot 6H_2O$, 0.3 mg; $CuSO_4 \cdot 5H_2O$, 0.3 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.4 mg; $CaCl_2 \cdot 2H_2O$, 4.5 mg; $FeSO_4 \cdot 7H_2O$, 3 mg; H_3BO_3, 1 mg; KI, 0.1 mg; Uracil, 25 mg and 0.1 ml of a 10% solution of silicone antifoam. Vitamin concentrations per liter were: D-Biotin, 0.05 mg; Ca-D (+) Panthothenate, 1 mg; Nicotinic acid, 1 mg; *myo*-Inositol, 25 mg; Thiamine hydrochloride, 1 mg; Pyridoxine hydrochloride, 1 mg and *p*-amino benzoic acid, 0.2 mg.

Batch and continuous cultivation conditions

Batch growth of the yeast cells was studied in a laboratory shaker incubator at 30°C and 200 rpm. A loopful of cells collected from YPD agar plates were directly used to inoculate the defined mineral medium in shake flask culture which resulted in a low initial cell density in the culture (A_{600} = 0.01). The initial concentrations of P_i in the culture media were set at 0, 6, 11, 23, 107, 222, 550, and 972 μM.

In continuous cultivation, yeast cells were grown at 30°C in a chemostat culture under P_i limitation using a laboratory bioreactor (Biostat B, B. Braun Biotech International, Germany) at a stirrer speed of 700 rpm. Yeast cells collected from YPD agar plates were used to inoculate an agitated preculture containing synthetic medium with a composition identical to that used for batch and continuous cultivation media except for the omission of KCl and an increased P_i concentration of 3 g l^{-1}. Cells were grown at 30°C and 200 rpm for 24 h, harvested by centrifugation at 5,000×*g* for 5 min, washed with sterile water, and resuspended in defined, synthetic medium for inoculation of the bioreactor. The amount of inoculation was 1% (vol.) of the growth medium (Initial A_{600} = 0.05). The initial concentration of P_i in the bioreactor was at the level of 250 μM and the concentration of P_i in feed stream was set to 1 mM. Following batch growth of the cells in a P_i-limited (250 μM P_i) medium until all P_i was consumed, the feed pump was started to allow for operation in continuous mode. The chemostat culture volume was maintained at approximately 1.0 l. The exact working volume was measured after each experiment. The pH was kept at 4.5 by automated addition of 2 M KOH. A constant airflow rate of 5 l min^{-1} was achieved by a flowmeter. The dissolved oxygen concentration of the culture was monitored with an O_2 electrode (InPro 6000 Series, Mettler Toledo) and remained above 60% of air saturation during the cultivation. This level of oxygen saturation, i.e. above the critical level, ensured that aerobic conditions prevailed in all experiments [10].

Different dilution rates (D) of the chemostat culture were achieved by changing the speed of the external variable-speed peristaltic pump (model 403U/L2, Watson-Marlow-Alitea, England). After each increase of D, cultures were allowed to establish a new steady state involving at least five media volume exchanges after the prior change of growth conditions, a situation at which the optical density of the culture monitored at 600 nm (A_{600}), biomass concentration, P_i and glucose concentrations had remained constant for at least two volume changes [10]. Replacing the feed medium by a 4 mM P_i solution after establishment of steady state condition using a feed solution of 1 mM P_i concentration resulted in an increased trend in biomass formation. This test verified the P_i-limited condition of the chemostat. In order to make sure that the chemostat was P_i-limited rather than subjected to a dual P_i-glucose limitation, a high level of glucose (30 g l^{-1}) was used in the feed medium. Under these conditions, glucose concentrations remained at adequate levels at very low dilution rates (0.08 and 0.1 h^{-1}) while P_i was almost exhausted at the same dilution rates (see Table 1). These findings proved that the P_i concentration by itself exerted limitation on the chemostat culture.

All data reported for continuous cultivation mode are from cultures in physiological steady state. Culture purity was monitored under microscope and by plating on YPD agar medium.

Analytical methods

Cell growth was monitored spectrophotometrically by measuring the absorbance of the culture at 600 nm (A_{600}). Biomass concentrations were measured using the method of oven drying of the cells [17]. Glucose and ethanol in supernatants were determined using glucose oxidase (Boehringer Manneheim) and ethanol (Boehringer Manneheim/R-Biopharm) enzymatic kits,

Table 1 Residual phosphate and glucose, ethanol and biomass concentrations in chemostat culture of *S. cerevisiae* CEN PK.113-5D/Pho84-C*myc* under different dilution rates

Dilution rate (h^{-1})	Phosphate (μM)	Glucose (mM)	Ethanol (mM)	Biomass (g l^{-1})
0.08	5	1.2	78.3	4.9
0.1	9	3.9	56.5	4.9
0.22	65	39.9	132.6	2.4
0.25	62	31.1	139.1	2.4
0.35	76	74.4	146.3	2.0
0.45	117	67.2	107.4	1.6

respectively. Extracellular P_i concentration was determined spectrophotometrically as described previously [39].

Growth rates (μ) were calculated from a semi-logarithmic plot of A_{600} versus time (based on equation of growth rate, Eq. 1). Growth rates were taken to be equal to the slope of the exponential phase of the growth curve. For very low P_i concentrations, since from the onset of the exponential phase the growth rate significantly changes with P_i concentration, initial rate data were determined.

$$A_{600} = a\exp(\mu t). \quad (1)$$

In situ specific P_i consumption rates at each dilution rate of chemostat culture were calculated from a mass balance of the bioreactor at that dilution rate (Eq. 2) [28].

$$V_{\text{insitu}} = \frac{(P_{\text{if}} - P_i)D}{w}. \quad (2)$$

In this equation, P_{if} represents P_i concentration in feed stream (μM), P_i is residual P_i concentration in the culture (μM), w is cell dry weight (g l^{-1}), and D is dilution rate (h^{-1}). Specific rates of glucose consumption and ethanol production at each dilution rate were calculated by the identical method as described above.

Electrophoresis and western blot analysis

Yeast cells were collected by centrifugation at 5,000×*g*, 4°C for 5 min, followed by extraction of proteins [34]. Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis using a 10% polyacrylamide and bispolyacrylamide gel system [14]. The electrophoretically separated proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) and immunoblotting was carried out according to the Western blotting protocol (Amersham Biosciences). Use of epitope-specific anti-*myc* antibody and horseradish peroxidase-conjugated anti-mouse-Ig (sheep) antibody allowed for immunological detection of the Pho84-C*myc* protein.

After a short incubation with chemiluminescent substrates, the blot was exposed to film for 1–2 min. The molecular mass of separated proteins was estimated by the relative mobilities of prestained marker proteins (Bio-Rad).

Phosphate transport assay

Cells were harvested by centrifugation at 5,000×*g*, 4°C for 5 min, washed once in ice-cold 25 mM Tris–succinate buffer (pH 5.5), and resuspended in 25 mM Tris–succinate (pH 5.5) containing 3% glucose, to a concentration of approximately 15–50 mg ml^{-1}.

Pho84 activity was assayed as described previously [39] by the addition of 1 μl ^{32}[P]orthophosphate to 30 μl aliquots of the cell suspension to a final concentration of P_i of 0.11 mM.

Results and discussion

The growth kinetics of *S. cerevisiae* strain CEN.PK113-5D/Pho84-C*myc* in batch and P_i-limited chemostat cultivation

Batch cultivation

Figure 1 shows the effect of P_i concentrations on the growth of yeast cells in batch culture. The results presented in Fig. 1a show that the lengths of lag phases are independent of initial P_i concentration whereas the slope of the exponential phase becomes steeper as the initial concentration of P_i is increased.

In Fig. 1b the relationship between the yeast growth rate and P_i concentration is presented. There was a hyperbolic relationship between the specific growth rate and the extracellular substrate concentrations. A Hanes–Woolf plot of the growth rate versus P_i concentrations data presented in Fig. 1c clearly shows that, growth kinetics follows the Monod relationship. K_S and $\mu_{\max}$ values calculated from this plot were 22 μM and 0.47 h^{-1}. It should be pointed out that the calculation of K_S values from results of batch culture is not very accurate, but this method accurately calculates maximum specific growth rate ($\mu_{\max}$) [11]. Therefore, K_S values obtained thus should only be used for comparative purposes.

The calculated $\mu_{\max}$ shows a good agreement with the values previously reported for aerobic and anaerobic cultivation of *S. cerevisiae* cells [9].

Another result taken from growth characteristics of cells as a function of P_i concentration in shake flask experiments is that, as long as the P_i concentration remains below 150 μM, the growth rate will be lower than $\mu_{\max}$ and it can therefore be controlled by the dilution rate in chemostat runs. This finding has been used in setting the P_i-limited conditions of continuous cultivations.

Continuous cultivation

Dilution rates in the range of 0.08–0.45 h^{-1} were used throughout this study. The steady state concentrations

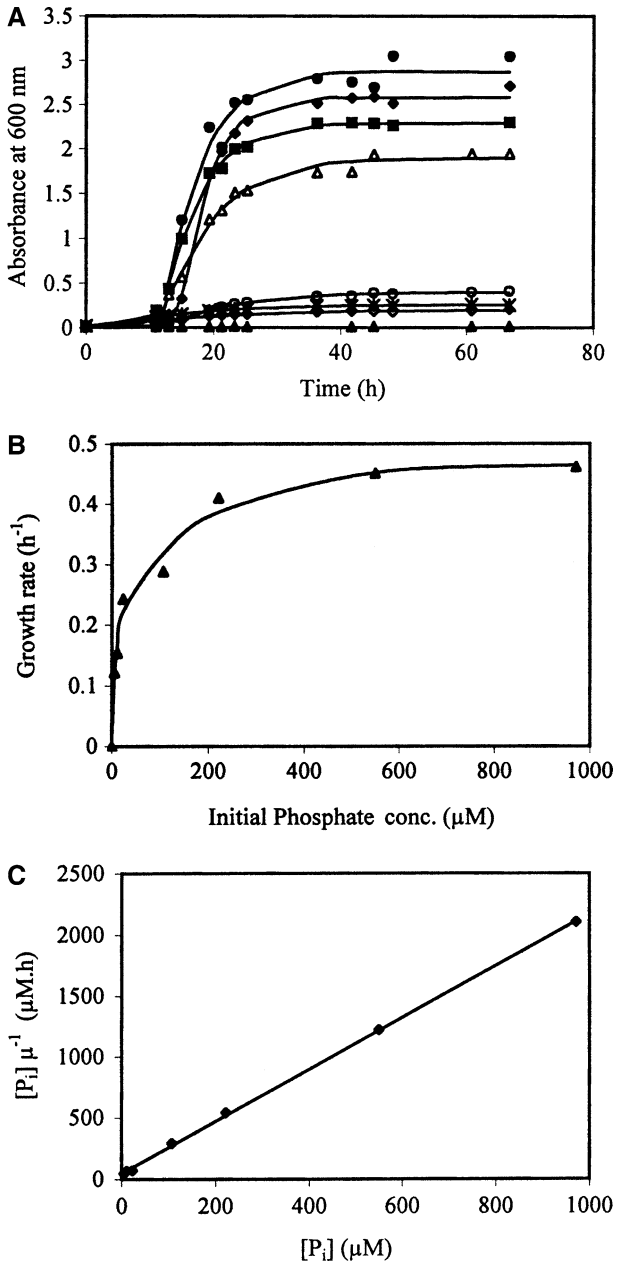

Fig. 1 Growth of *S. cerevisiae* cells in batch culture. **a** Influence of initial phosphate concentration on growth of the cells. Phosphate concentrations were: 972 µM (*filled circle*), 550 µM (*filled lozenge*), 222 µM (*filled box*), 107 µM (*open triangle*), 23 µM (*open circle*), 11 µM (*star*), 6 µM (*open lozenge*) and 0 µM (*filled triangle*). **b** Relationship between growth rate and phosphate concentration. **c** Hanes–Woolf kinetic plot of growth rate and phosphate concentration

of residual P_i, glucose, ethanol, and biomass concentrations are presented in Table 1. An increase in the dilution rate from 0.08 to 0.45 h^{-1} resulted in an increase in steady state P_i and glucose concentrations. While P_i was almost completely consumed at low dilution rates (0.08 and 0.1 h^{-1}) the available P_i concentration of the medium increased to 117 µM at conditions of a high dilution rate (0.45 h^{-1}).

Attempts at correlating the growth rate data obtained at the various dilution rates in this study according to Monod kinetics was unsuccessful. By use of the Monod equation, residual media P_i concentrations as a function of applied dilution rates (0.08–0.45 h^{-1}) are shown in the Eadie–Hofstee plot (Fig. 2). The data of Fig. 2 reveals that the cells' growth apparently obeys Monod kinetics only at low dilution rates (0.08–0.22 h^{-1}). The striking change in growth kinetics of the strain at dilution rates higher than 0.22 h^{-1} clearly indicate that other parameters than residual P_i contribute to the culture control of the growth rate and/or kinetic parameters of Monod model change by increasing dilution rate.

In Fig. 3 the growth rate obtained under batch cultivation at various P_i concentrations is compared with the corresponding data obtained from chemostat experiments. It should be noted that when the effect of P_i on growth is studied in batch culture, the cells have a transient experience of each P_i concentration whereas this is not true during chemostat cultivation, which may well result in accumulation of P_i in from of polyphosphate (PolyP) reserves at higher extracellular P_i concentrations (which corresponds to higher D).

Deviations from Monod kinetics with increase in the dilution rate above a certain value has previously been reported for aerobic and anaerobic glucose-limited chemostat cultivation of *S. cerevisiae* [9]. In the P_i-limited chemostat cultivations, increase in dilution rates corresponds to an increased accumulation of ethanol and P_i in the extracellular environment. Both these factors influence nutrient uptake kinetics. For example, De Kock et al. [8] postulated an interaction

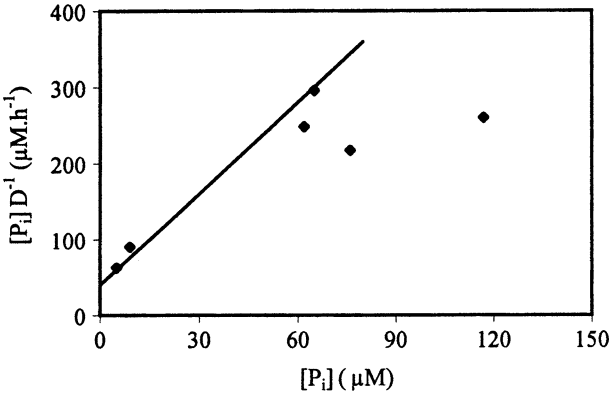

Fig. 2 Steady state phosphate concentration (*filled lozenge*) as a function of dilution rate in chemostat culture of *S. cerevisiae* cells in form of Hanes–Woolf plot

between ethanol concentration and activity of glucose transport proteins whereas P_i in the form of PolyP reserve has an enhancing effect on the high-affinity P_i transport [1, 5, 26].

The growth rate of cells at dilution rates exceeding 0.22 h^{-1} might be controlled by intracellular rather than extracellular P_i. Some cases have been reported in the literature where growth rates of chemostat cultures of so-called ''conservative'' substrates (P_i, K, Mg) is independent of the culture concentration of the limiting nutrient [22]. For instance, in the case of P_i-, K- or Mg-limited cultures of *Aerobacter aerogenes* it was concluded that the growth rate was a linear function of the intracellular concentration [22].

Another explanation of this change in growth pattern would be changes in metabolism of the cells, as an example biphasic growth kinetics of glucose-limited chemostat cultures of *S. cerevisiae* has previously been reported [30].

Culture behavior and cellular changes with dilution rate

Figure 4 shows some parameters that characterize the metabolic activities of *S. cerevisiae* strain CEN.PK113-5D/Pho84-C*myc* in the P_i-limited chemostat culture for dilution rate in the range 0.08–0.45 h^{-1}. The results show that the rates of nutrient uptake and metabolite production are a function of the applied dilution rate. Increase in the dilution rate in the range 0.08–0.45 h^{-1} resulted in an increase in both the P_i and glucose consumption rates and a 23-fold increase, from 1.3 to 30.2 mmol g dry wt^{-1} h^{-1}, in the specific rate of ethanol production. The highest biomass yield coefficient based on both P_i ($Y_{X/P}$) and glucose ($Y_{X/G}$) consumed, was obtained at $D = 0.1$ h^{-1} (30.5 g gP_i^{-1} and 0.15 g $glucose^{-1}$). However, increase in dilution rate resulted in a general decrease in the yield coefficients.

Results presented in Fig. 4a also show an increase in the rate of P_i uptake by the cells (Q_{P_i}) with increasing dilution rate. These results indicate that the capacity of this organism to accumulate P_i is not saturated in the range of dilution rates used in this study.

The fact that with increasing dilution rate in the range 0.08–0.45 h^{-1}, biomass yield coefficient based on P_i decreases whereas Q_{P_i} increases seems to suggest that at higher dilution rates part of the P_i is utilized in the production of intracellular reserves such as the PolyP pool. Similar behavior has been observed for magnesium-limited chemostat cultures of *S. cerevisiae* [35] as well as for P_i-limited chemostat cultures of some algal and bacterial cells [22].

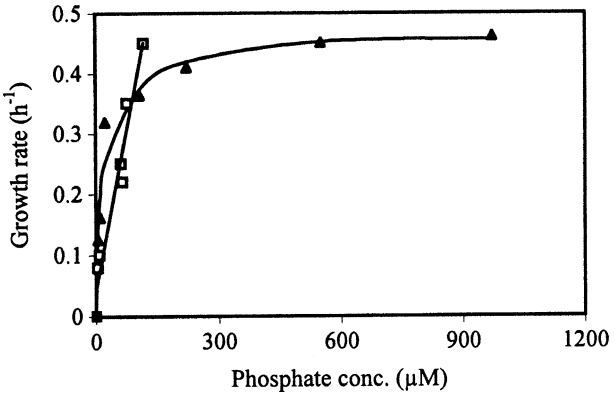

Fig. 3 Dependency of growth rate/dilution rate on the extracellular phosphate concentration in chemostat culture (*open box*) and batch cultivation of *S. cerevisiae* cells (*filled triangle*)

Microbial performance is commonly assessed using the specific consumption and/or production rates of substrates or metabolites. Figure 4b shows that at higher dilution rates (which correspond to higher glucose concentrations) a higher specific glucose consumption and ethanol production rate and a lower $Y_{X/G}$ is obtained under the same growth condition. Changing biomass yield with changes in dilution rates in glucose-limited growth of *S. cerevisiae* was previously reported to be due to a lower respiratory activity at high glucose concentrations [10, 29]. Higher specific glucose consumption and ethanol production rates, increased ethanol yield coefficient, as well as the decrease in biomass yield on glucose, which accompany the increase in the dilution rates, seem to indicate an increased metabolic flux through glycolythic pathway leading to ethanol.

Activity and expression of the Pho84 transporter in chemostat culture

The dependence of growth characteristics of the living cells and nutrient transport to them by the aid of transporters is obvious. Although the relationship between growth parameters and P_i-transport activity of *S. cerevisiae* cells has been previously shown in batch mode [19, 27], there is no report available on P_i transport behavior in chemostat culture.

In order to study the expression of P_i transporters at the conditions employed in this investigation, samples from the bioreactor during chemostat cultivation of *S. cerevisiae* strain CEN.PK113-5D/Pho84-C*myc* at dilution rates equal to 0.08, 0.1, 0.22, 0.25, 0.35 and 0.45 h^{-1} were subjected to electrophoresis and western blot analysis. Since the Pho84 transporter

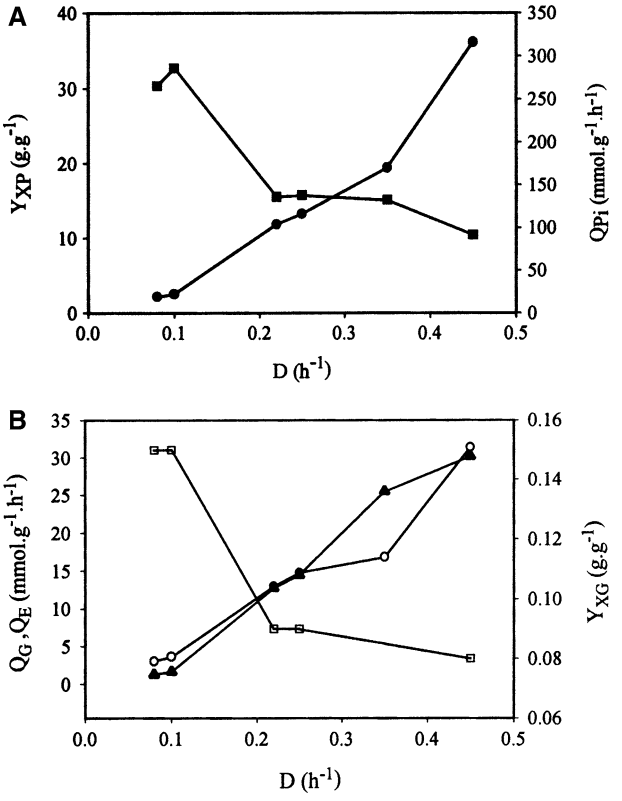

Fig. 4 Growth and metabolic parameters of *S. cerevisiae* CEN.PK 113-5D in a P_i-limited chemostat culture at various dilution rates. **a** Biomass yield on phosphate, Y_{XP} (*filled box*), and specific rate of phosphate consumption, Q_{P_i} (*filled circle*) versus dilution rate. **b** Biomass yield on glucose, Y_{XG} (*open box*), specific rate of glucose consumption, Q_G (*open circle*), and specific rate of ethanol production, Q_E (*filled triangle*) versus dilution rate

is the major P_i uptake system operative under low P_i conditions [26], the expression of this protein was measured using western blot analysis. The results, together with the corresponding data for P_i concentrations and activity of Pho84 high-affinity transporter obtained from ^{32}[P] uptake measurements, are presented in Fig. 5. Expression of Pho84-*myc* protein was evaluated by western blot analysis of isolated plasma membrane from *S. cerevisiae* cells using suitable antibodies.

The resulted Pho84 protein band of western blot analysis is also shown in Fig. 5. The result (Fig. 5, 35-kDa protein band) shows the expression of Pho84, occurred at all dilution rates tested in the present study. The intensive protein bands at dilution rates of 0.08 and 0.1 h^{-1} clearly shows higher amount of Pho84 expressed at these dilution rates, which is corresponds to very low P_i concentration (9 μM), while lower expression of this transporter was seen at higher dilution rates.

As can be seen in Fig. 5, Pho84 had its highest activity as well as highest level of synthesized protein at the lower range of the applied dilution rates (6.14 and 5.37 μmol g dry wt^{-1} min^{-1} for dilution rates of 0.08 and 0.1 h^{-1}, respectively). Under these conditions, the external P_i concentrations were lower than 10 μM. This result is consistent with earlier studies that have shown that the majority of P_i is transported by Pho84p at low P_i concentrations [19]. However, previous results obtained through batch studies of *S. cerevisiae* cells grown in low-P_i YPD media have shown that synthesis and activation of the Pho84 high-affinity P_i transporter occurs exclusively at external P_i levels in the range 20–70 μM under conditions of non-limiting carbon supply [15, 19]. Our results suggest the expression of high-affinity phosphate transporters in the chemostat at P_i concentrations (< 20 μM), which in previous shake flask (batch) studies were found to be repressible P_i concentrations. This difference can probably be attributed to the difference in the environmental conditions experienced by cells when grown in batch and chemostat modes, although the partial effect of the difference in the strains and media used in the previous batch cultivations compared to the chemostat study can not be ruled out. During batch cultivation, the yeast cells experience a transient environmental condition as far as the concentration of nutrients is concerned. On the other hand during chemostat cultivation, after steady state is achieved, the cells experience constant concentration of nutrients in their environment. Therefore, the cell's composition and physiological state will change during the batch experiments, whilst, after attainment of steady state, it remains unaltered during chemostat cultivation. It seems that under the latter conditions high-affinity P_i transporters are expressed at lower P_i concentrations compared to when cells are cultivated under transient nutrient concentrations.

This de novo synthesis of Pho84 is the result of a rapid response of the *PHO* pathway regulated gene expression in cells meeting a P_i limitation. However, at P_i concentrations approaching the K_m of the transporter Pho84-mediated transport is abolished and the protein routed to the vacuole for degradation [15, 19, 27]. An increment of the dilution rates to 0.22 and 0.25 h^{-1} corresponding to an external P_i concentration of 60–65 μM resulted in decreased levels of synthesized Pho84 and an at least 13-fold decrease in Pho84-mediated P_i uptake activities to 0.41 and 0.28 μmol g dry wt^{-1} min^{-1}, respectively. This may be accounted for by a decreased concentration of Pho84 in the plasma membrane (Fig. 5). Interestingly, an increase of the dilution rates to 0.35 and 0.45 h^{-1}, corresponding to

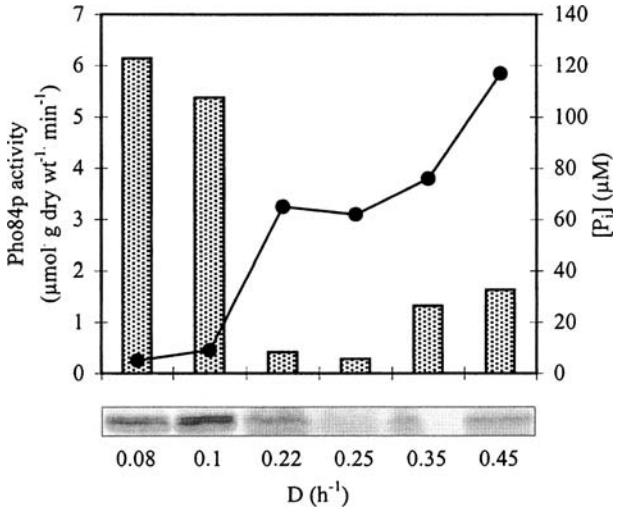

Fig. 5 The activity of Pho84 high-affinity transporter obtained from ^{32}[P] uptake measurements (*bars*), P_i concentration (*filled circle*) and western blot analyses (*box under the abscissa*) of Pho84-C*myc* of cells taken from P_i-limited chemostat cultures operating at different dilution rates

an external P_i concentration of 76 and 117 μM, respectively, resulted in increased level of synthesized protein paralleled by a fivefold increase of the Pho84p-mediated P_i uptake activity (1.32–1.63 μmol g dry wt^{-1} min^{-1}). The regulatory mechanism allowing for the recovered synthesis and activation of the Pho84 under these conditions is not known but may be due to a shift in the metabolism of the cells and P_i transport behavior at dilution rates higher than 0.22 h^{-1}.

Our results on P_i-limited chemostat growth of the CEN.PK113-5D strain presented here strongly support the expression of Pho84 at a P_i concentration range of 5–117 μM. The changing nutrient concentrations as well as accumulation of metabolites encountered in batch cultivation compared to chemostat experiments could be possible reasons for the lower repressible P_i concentration reported in the former studies.

It is interesting to note that all observed phenomena in continuous studies, namely deviation from or change in the parameters of Monod kinetics, decrease in biomass yield coefficients on glucose and phosphate, increase in ethanol yield, significant decrease in expression and activity of Pho84 transporter all happen at dilution rate of around 0.2 h^{-1}. These phenomena could be the consequence of the change in the nutrient concentrations in the internal and external environment of the yeast cells when the dilution rate is increased above 0.2 h^{-1}. There is a significant difference between P_i and glucose concentrations at $D < 0.22$ h^{-1} and $D \geq 0.22$ h^{-1} (9 vs. 65 μM and 3.4 vs. 39.9 mM, respectively). As discussed before, increased P_i concentration could have led to accumulation of P_i within the yeast cells. Increased glucose concentration, on the other hand, could have resulted in the Crabtree effect. However, it should be pointed out that when Crabtree effect happens, biomass yield on glucose decreases, rate of glucose consumption increases significantly, and significant concentrations of ethanol start appearing in the media [10, 29]. In our experiments although biomass yield on glucose did decrease significantly when D was raised above 0.2 h^{-1}, but there was no sharp change in the rate of glucose consumption and significant ethanol concentrations were detected throughout the entire range of dilution rate tested.

Acknowledgments We gratefully acknowledge Prof. Bengt L. Persson (Kalmar and Stockholm Universities, Sweden) for acting as a co-advisor for the first author in her PhD program and for providing laboratory facilities and yeast strain. We are deeply grateful for the valuable assistance of Dr. Jens O. Lagerstedt (University of California-Davis, USA) in the western blot analyses. The authors are grateful for financial support from The Ministry of Science, Research and Technology of Iran, the KK Foundation, Human Frontier Science Organization, the Swedish Natural Science Research Council, and Växjö University, Sweden. Technical assistance of Helena Rupar is acknowledged.

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