Characterization of Phosphate Membrane Transport in *Saccharomyces cerevisiae* CEN.PK113-5D under Low-Phosphate Conditions Using Aerobic Continuous Culture

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ABSTRACT: *Two different growth media, namely complex and defined media, were used to examine establishment of steady-state conditions in phosphate-limited culture system of Saccharomyces cerevisiae CEN.PK113-5D strain. Using the defined growth medium, it was possible to obtain steady state condition in the continuous culture. The effect of phosphate concentration on the growth of S. cerevisiae in phosphate-limited chemostat was studied at dilution rates between 0.08-0.45 h^{-1}. The cells' growth followed Monod kinetics only over low dilution rates (0.08-0.22 h^{-1}) in which the saturation constant (K_S) and maximum growth rate (μ_m) were determined as 10 μM and 0.25 h^{-1}, respectively. By increasing the dilution rates above 0.22 h^{-1}, a significant change in the growth pattern was occurred, possibly due to intracellular accumulation of phosphate and/or extracellular accumulation of ethanol and also increased fermentative activity of the yeast cells.*

Phosphate transport of the yeast cells via plasma membrane transporters was kinetically characterized in a phosphate-limited chemostat culture. The rate of phosphate transport was measured using ^{32}[P]-labeled orthophosphate in the concentration range of 0.4-2000 μM. High-affinity phosphate transport kinetics was observed over the entire range of dilution rates tested in this study. The corresponding K_m values for phosphate were found to be in the range of 1.7 to 36 μM. Dilution rate of 0.22 h^{-1} showed biphasic pattern for phosphate uptake kinetics while the estimated K_m values for this behavior were 1.7 and 284 μM.

KEY WORDS: *Cell membrane transport, Kinetics of phosphate transport, Saccharomyces cerevisiae, Chemostat culture, Yeast's growth kinetics.*

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1021-9986/05/1/41 11/$/3.10

INTRODUCTION

Almost all nutrients enter the cell *via* transport proteins whose capacity and affinity properties vary. The synthesis and activity of such proteins may thus be modulated by changes in the extracellular environment as part of an adaptive response. In most cases, kinetics of membrane transport of nutrients follows Michaelis-Menten model [1].

Orthophosphate ($H_2PO_4^-$) plays an important role in cell functioning, being involved in most metabolic energy transductions and serving as an intermediate in the biosynthesis of numerous metabolites.

Phosphate (P_i) metabolism in the yeast *S. cerevisiae* has been extensively studied for many years and this unicellular eukaryote has provided an excellent model system for understanding of how a cell makes a coordinated response to environmental P_i changes (for recent reviews, see [1-3]).

P_i transport across the plasma membrane of *S. cerevisiae*, is mediated by several specific plasma membrane transport systems allowing the cell to switch between different, low affinity and high-affinity, modes of P_i acquisition. The high-affinity phosphate transport system, expressed upon P_i starvation during aerobic and anaerobic growth, is active under low-phosphate (LP_i) conditions in the presence of an abundant carbon source [4-6]. The high-affinity characteristic of the transport is reflected by a K_m for P_i in the range of 1-45 μM [7-9]. From the two high-affinity P_i transporters, one is the gene product of *PHO84* (Pho84p) [4] which catalyzes a H^+-coupled P_i transport [10-12] and the other is a cation-coupled P_i transporter (Pho89p) encoded by the *PHO89* gene [6,8]. At non-limiting, repressive P_i conditions, a low-affinity transport system with a K_m for P_i in the range of 0.8-1 mM has been shown to be active [7,13]. Recently, the overexpression of *PHO87*, *PHO90*, or *PHO91* gene was shown to lead to an increase in P_i uptake ability suggesting a role of Pho87p, Pho90p and Pho91p as components of the low-affinity P_i transport system [14]. Pho84p is responsible for the majority of P_i uptake into the cells while the contribution of Pho89p, Pho87p, Pho90p and Pho91p appear to be lower [8,14-16].

However, so far, essentially all characterization of P_i transport in *S. cerevisiae* has been carried out using cells grown in shake flasks on complex YPD-based growth media. These flask culture experiments suffer from the disadvantage that several environmental factors such as *p*H, P_i concentration and oxygen tension are not maintained constant during the course of the experiments and hence the cell's composition and physiological state change during the experiment (for a review, see [17]). The continuous cultivation (chemostat) technique allows for a detailed study of specific environmental conditions, since each parameter can be changed independently [18-20]. Transport kinetics and regulation of transporters such as hexose and amino acid transporters have been extensively studied by the chemostat technique [21-24]. Despite these extensive research works, no studies concerning P_i membrane transport of *S. cerevisiae* under P_i-limited growth in continuous cultures are reported in the literature.

Several studies have been reported on nutrient-limited growth of *S. cerevisiae* [25-29]. Many models of the aerobic batch and continuous growth of *S. cerevisiae* have already been published. Most of them are structured models which have been proposed for glucose-limited growth of the yeast cells [30,31]. The growth of some *S. cerevisiae* strains in both aerobic and anoxic chemostat cultures have been reported to deviate from Monod kinetics [32-34]. This has been attributed to some growth factor deficiency when the dilution rate is increased above a certain level, which could result in a switch from a respiratory to respirofermentative metabolism or to increased accumulation of ethanol in the fermentation broth. The kinetics of growth of some yeast, bacterial and algal cells on P_i have been studied using chemostat cultivation and in some cases the growth rate has been correlated with the internal and sometimes the external P_i concentration [25,26,35,36]. However, there is no report available on P_i-limited growth of *S. cerevisiae* cells.

We have here characterized P_i-limited growth and phosphate transport of steady-state cultures of *S. cerevisiae* strain CEN.PK113-5D under low-P_i concentrations using chemostat cultivation technique.

MATERIALS AND METHODS

Materials

^{32}[P]orthophosphate (carrier-free) was obtained from Amersham-Pharmacia Biotech, Sweden. D-glucose and ethanol enzymatic bioanalysis kits were obtained from Boehringer-Manneheim/R-Biopharm, Sweden. All other

materials were of analytical grade and obtained from commercial sources.

Yeast strain and growth media preparation

Saccharomyces cerevisiae strain CEN.PK113-5D (*MAT*a *MAL2-8c SUC2 ura3-52*) has been used in this study [37]. This strain is auxotrophic derivative of the haploid, prototrophic *S. cerevisiae* CEN.PK113-7D (*MAT*a *MAL2-8c SUC2*). Cells were grown on YPD agar plates (1% yeast extract, 2% peptone, 2% glucose and 2% agar) and maintained at 4°C.

The culture media used for aerobic cultivation of CEN.PK113-5D cells were either a YPD complex medium (1% yeast extract, 2% peptone and 2% glucose) in which the phosphate content was reduced by precipitation [38] yielding a low phosphate-YPD medium, or a defined, synthetic mineral medium prepared essentially as described by Verduyn et al. [39] but with minor modifycations in the composition of main elements. Composition of main elements were: Glucose, 30 g. L^{-1}; $(NH_4)_2SO_4$, 5 g. L^{-1}; $MgSO_4.7H_2O$, 0.5 g. L^{-1}; KH_2PO_4, 250-1000 µM and KCl, 24.1-23.4 mM (The concentration of P_i in starting batch cultivation and feed reservoir of defined medium were 250 and 1000 µM, respectively. The corresponding concentrations of KCl in these media were 24.1 and 23.4 mM, respectively). Trace elements additions per liter were: EDTA, 15 mg; $ZnSO_4.7H_2O$, 4.5 mg; $MnCl_2.4H_2O$, 1 mg; $CoCl_2.6H_2O$, 0.3 mg; $CuSO_4.5H_2O$, 0.3 mg; $Na_2MoO_4.2H_2O$, 0.4 mg; $CaCl_2.2H_2O$, 4.5 mg; $FeSO_4.7H_2O$, 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. Vitamins 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.

While the main element solution was sterilized by autoclaving for 40 min at 110 ºC, vitamin and trace element solutions were sterilized by 0.2 µ filtration. Glucose solution was autoclaved separately (30 min at 110 °C) and subsequently added to the bioreactor together with the trace element and vitamin solutions.

Continuous cultivation conditions

Yeast cells were grown at 30 °C in a continuous culture under P_i-limitation using a laboratory bioreactor (Biostat B, B. Braun Biotech International, Germany) at a stirrer speed of 700 rpm. Some of the specifications of the fermentor are: stirrer speed range, 50-1200 rpm; jacketed heating system; maximum working volume, 2 L; height/ diameter ratio of the vessel, about 2:1. Yeast cells collected from YPD agar plates were used to inoculate an agitated preculture containing defined, synthetic media with a composition identical to that used for the chemostat medium except for the omission of KCl and an increased concentration of P_i to 3 g. L^{-1}. Cells were grown in shake flasks at 30°C and 200 rpm for 24 h, harvested under sterilized conditions by centrifugation at 5,000 x g for 5 min, washed with sterile water, and resuspended in growth medium for inoculation of the fermentor. The amount of inoculation was 1% (vol) of the growth medium. 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 400 µM and 1 mM for YPD and defined media, respectively. After cells were grown batchwise in a P_i-limited (250 µM P_i) medium and by time all P_i was consumed, the operation in continuous mode was started by initiating work of the feed pump. The chemostat culture volume was maintained at approximately 1.0 liter by a peristaltic effluent pump coupled to a level pipe. The exact working volume was measured after each experiment. The *p*H was kept at 4.5 by automated addition of 2 M KOH. A constant air flow rate of 5 L. min^{-1} was maintained appropriately using 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 [21,40,41].

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 (OD_{600}), biomass concentration, P_i and glucose concentration had remained constant for at least two volume changes [21,42].

Culture purity was routinely monitored under microscope (magnification of 1000) and by plating on YPD agar medium.

All data reported for continuous cultivation mode are from cultures in physiological steady state.

Analytical methods

Cell growth was monitored spectrophotometrically by measuring the absorbance of the culture at 600 nm (OD_{600}). Biomass concentrations were measured using the method of oven-drying of the cells [27]. Glucose and ethanol in supernatants were determined using glucose oxidase (Boehringer Manneheim) and ethanol (Boehringer Manneheim / R-Biopharm) enzymatic kits, respectively. Extracellular P_i concentration was determined spectrophotometrically as described previously [43].

Phosphate transport assay and its kinetic determination

Cells were harvested by centrifugation at 5,000 x g, 4°C for 5 min, washed once in ice-cold 25 mM Tris-succinate buffer (*p*H 5.5), and resuspended in 25 mM Tris-succinate (*p*H 5.5) containing 3 % glucose, to a concentration of approximately 15-50 mg ml^{-1}. For the kinetic analyses, P_i uptake in cells harvested at each steady state condition was assayed as described previously [43] by the addition of 1 µl ^{32}[P]orthophosphate (carrier-free, 0.1Ci/µmol, 1 mCi =37 MBq) to 30 µl aliquots of the cell suspension to final P_i concentrations ranging from 0.4 to 2000 µM. Initial rates of phosphate uptake (µmol P_i transported. g dry wt^{-1}. min^{-1}) were calculated from the slope of the initial linear part of the uptake curve (*zero-trans method*). Average values are based on triplicate determinations performed at each concentration.

The transport kinetic parameters, K_m and V_m, estimated from the *zero-trans* influx measurements were fitted to Michaelis-Menten kinetic model (Eq. 1) using Hanes-Woolf plot (Eq. 2). A plot of $[P_i]/V$ versus $[P_i]$ results in a line of slope $1/V_m$ and y-axis intercept of K_m/V_m. The coefficients of correlation values (R^2) for linear regression of the reciprocal plots exceeded 0.96 in all experiments.

$$V = \frac{V_m [P_i]}{K_m + [P_i]} \quad (1)$$

$$\frac{[P_i]}{V} = \frac{1}{V_m}[P_i] + \frac{K_m}{V_m} \quad (2)$$

K_m denotes the apparent affinity constant of transport system (µM), V_m is the maximum capacity of transport system (µmol. g dry wt^{-1}. min^{-1}), $[P_i]$ is residual phosphate concentration in the culture (µM) and V is specific rate of phosphate uptake (µmol. g dry wt^{-1}.min^{-1}).

RESULTS AND DISCUSSION

P_i-limited chemostat cultivation of the yeast cells provides a valuable tool to evaluate the relationship between the P_i-regulated physiological response of the cells and the transporter-mediated regulation of P_i uptake. In this study we have characterized growth, metabolic and transport activities of the *S. cerevisiae* CEN.PK113-5D strain at conditions of P_i-limitation.

Selection of growth media for P_i-limited continuous cultivation

Since previous characterization of the P_i transport systems in *S. cerevisiae* has been based on cells grown in YPD media containing 250-300 µM P_i using shake flask cultures [5,6,37], this medium was initially used in the present study. However, results obtained at D of 0.13 h^{-1} (Fig. 1A) and 0.23 h^{-1} (Fig. 1B) for *S. cerevisiae* show that steady state condition was initially established after 24 h but therefore there was a pronounced increase in P_i concentration with time (see Fig. 1 for details). It has previously been observed that low-P_i grown *S. cerevisiae* cells catalyze liberation of P_i resulting in a 2-fold increase of the external P_i concentration [6]. Periplasmically located phosphatases of the cells are the major enzymes active in scavenging P_i from available phosphorous compounds in the environment. During growth of *S. cerevisiae* in P_i-enriched media the synthesis and accumulation of P_i in the form of polyphosphate (PolyP) permits a polymer storage form of acquired P_i [1,18,44]. The PolyP pool can be degraded and used by cells grown under extended P_i-deficient conditions. Although, release of P_i might be due to disruption of the cells but this hypothesis was not supported by microscopic inspection in this study (Fig. 2).

In fact, measurements of polyphosphatase activity may help to get better understanding of this behavior. The obtained results indicated that YPD was not an appropriate medium for the continuous studies of P_i- limited cells

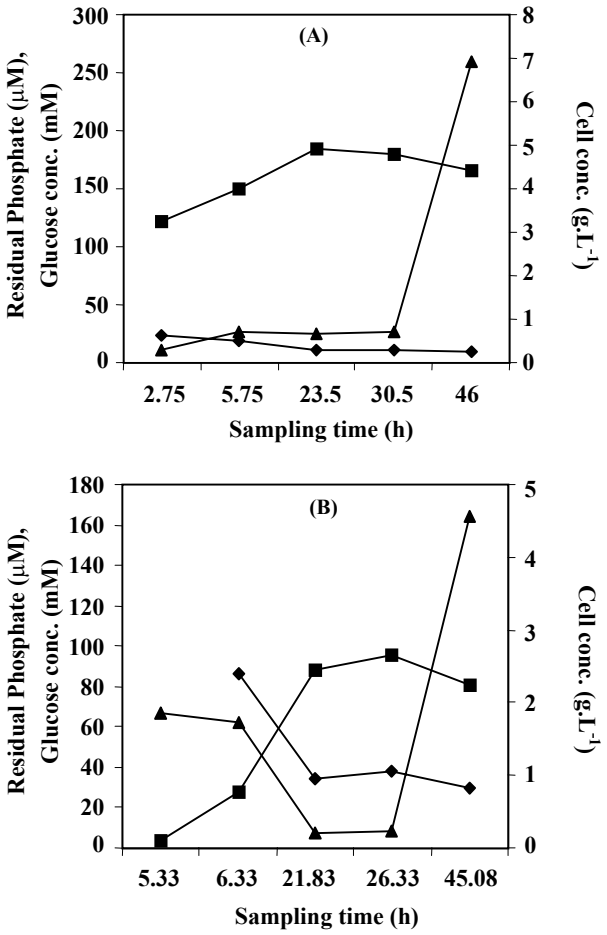

Fig. 1: Time course of phosphate and glucose utilizations and growth pattern of S. cerevisiae using YPD medium at dilution rates of 0.13 h^{-1} (A) and 0.23 h^{-1}(B). The feed was introduced to the fermentor at zero time. P_i and glucose concentrations in feed stream were 400 μM and 20 g. L^{-1}, respectively.
Symbols: ■, cell dry weight; ♦, glucose concentration; ▲, phosphate concentration.

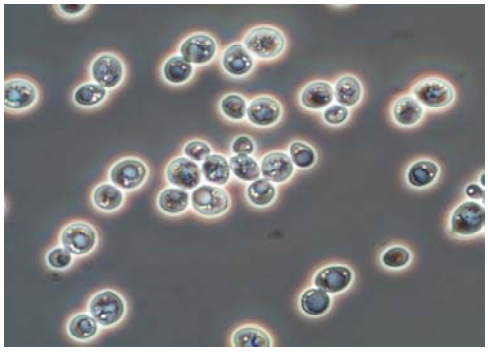

Fig. 2: Phase contrast microscopic image (x1000 magnification) of S. cerevisiae CEN.PK113-5D cell grown in P_i-limited continuous culture using YPD medium at dilution rate of 0.13 h^{-1}.

therefore; a defined medium was used, basically according to the specifications given by Verduyn [39]. Moreover, by using defined medium in chemostat studies the steady state conditions were established after 28 h and sudden changes in the P_i concentration were not detected (Table 1). Defined medium for growth of *S. cerevisiae* under P_i limitation was therefore used.

Measurement of the accessible P_i concentration in P_i-limited chemostat cultures

The P_i-dependent expression and activity of the P_i transporters of *S. cerevisiae* is a well documented characteristic of these cells [1,5]. In order to determine P_i transport kinetics in the chemostat culture, measurements of the available P_i concentration in the media as well as the rate of cellular P_i acquisition are necessary. A prerequisite for an accurate quantification of cellular metabolites (intra- and extra-cellular) is that there are no changes in concentration levels during the cell harvesting procedure.

Rapid measurements of the parameters to be investigated are required in order to prevent P_i utilization during the sample collection. The two alternative cell sampling techniques, rapid freezing in liquid nitrogen successfully used in glucose transport studies [21,23,45], and cell collection by centrifugation at 4 ^oC employed in previous P_i transport studies [5,37] were used in the present study. Table 2 shows the results obtained when cells grown at D=0.22 h^{-1} for 49, 52, and 55 h were harvested applying the two different harvesting techniques. The observed residual P_i concentration contained in the medium was consistently 2-4 fold higher when cells withdrawn were subjected to the rapid freezing technique as compared to cells collected by low temperature centrifugation. In contrast, the concentrations of residual glucose and ethanol produced in the cultures were close to identical independent of the harvesting method. A possible reason for the higher P_i concentration seen when the harvested cells were frozen in liquid nitrogen is that disruption of the membrane barrier results in a release of intracellular P_i and P_i storages, a possibility supported by visual inspection of the cells under microscope. Therefore, cell sampling by low temperature centrifugation was observed to be an appropriate harvesting method for the present P_i-limited chemostat study.

Table 1: Selected growth parameters for S. cerevisiae CEN.PK113.5D cultivated in P_i-limited chemostat system using defined medium at dilution rate of 0.22 h^{-1}.

Harvest time* (h)	OD_{600}	Cell dry wt. (g. L^{-1})	Residual P_i conc. (μM)	Residual glucose conc. (mM)
4.5	2.6	1.25	12.1	21.9
9.3	3.9	–	–	–
28	5.9	2.41	72.7	7.7
33	5.8	2.38	–	7.7
46	6.0	2.44	68.1	7.5
52	6.1	2.39	67.2	7.4

** Length of time after starting feeding to the system (see the text).*

Table 2: Comparison of the two cell harvesting methods used for P_i-limited chemostat culture at dilution rate of 0.22 h^{-1}. The samples were taken at 49, 52, and 55 hours after starting the chemostat cultivation.

Cell harvesting method	Phosphate conc. (μM)			Glucose conc. (g. L^{-1})			Ethanol conc. (g. L^{-1})		
	49 h	52 h	55 h	49 h	52 h	55 h	49 h	52 h	55 h
Freezing in liquid nitrogen	180.3	199.3	180.8	7.8	5.2	6.6	6.1	6.7	6
Centrifugation at low temp.	73.1	57.2	52.1	6.7	7.4	6.6	6.5	6.6	5.1

Table 3: Measurement of growth parameters in chemostat culture of S. cerevisiae CEN PK.113-5D under different dilution rates using defined medium. Phosphate concentration in feed stream was about 1 mM.

D (h^{-1})	P_i conc. (μM)	Glucose conc. (mM)	Ethanol conc. (mM)	Cell dry wt. (g. L^{-1})	Biomass yield on P_i (g. gP_i^{-1})	Biomass yield on glucose (g. g $glucose^{-1}$)
0.08	5	1.2	78.3	4.9	30.35	0.15
0.1	9	3.4	82.6	4.9	30.55	0.15
0.22	65	39.9	138.6	2.4	15.55	0.09
0.25	62	42.31	139.1	2.4	15.66	0.09
0.35	76	66.1	145.6	2.0	13.20	0.12
0.45	117	67.2	107.4	1.6	10.48	0.08

Growth behavior of S. cerevisiae in P_i-limited continuous culture

In this study, the P_i concentration in the feed reservoir was set at 1 mM in all chemostat experiments while the P_i concentration of the culture was adjusted by different dilution rates of the culture. Initially, results from batch culture grown *S. cerevisiae* cells were used to estimate the P_i concentrations necessary to impose growth limitations during the chemostat cultivation. Growth characteristics of cells as a function of P_i concentration obtained with shake flask experiments (not shown) revealed that, as long as the P_i concentration remains below 150 μM, the growth rate will be lower than μ_{max} and it can therefore be controlled by the dilution rate.

The range of dilution rates used in the present study was 0.08-0.45 h^{-1}. The steady state concentrations of residual P_i, glucose and ethanol, as well as biomass concentrations and biomass yields are presented in Table 3. Measurements of steady state P_i and glucose concentrations at various dilution rates showed that an increase in the dilution rate from 0.08 h^{-1} to 0.45 h^{-1} resulted in an increase in P_i and glucose concentrations. While P_i was almost completely consumed at low dilution rates (0.08 h^{-1} 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}).

By use of the Monod equation (Eq. 3) 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. 3).

$$D = \mu_m - K_S \frac{D}{[P_i]} \quad (3)$$

D denotes dilution rate (h^{-1}), μ_m is the maximum specific growth rate (h^{-1}) and K_S is the saturation constant for P_i (μM). The data of Fig. 3 reveals biphasic growth kinetics taken to indicate that the cells' growth apparently obeys Monod kinetics only at low dilution rates (0.08-0.22 h^{-1}). The saturation constant (K_S) of 10 μM was calculated from the slope of the plot. From the intercept with the Y-axis a μ_m of 0.25 h^{-1} was determined. These constants are in fairly good agreement with the growth parameters obtained in the shake flask experiments. 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.

A possible explanation for this might be that the growth rate of cells at dilution rates exceeding 0.22 h^{-1} is 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), not irreversibly consumed after uptake but stored within the cell, is independent of the culture concentration of the limiting nutrient [35,46]. 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 [35].

Deviations from Monod kinetics when dilution rate was increased above a certain value has previously been reported for aerobic and anearobic glucose limited chemostat cultivation of *S. cerevisiae* [34]. In the P_i-limited chemostat runs, increase in dilution rate corresponds to an increased accumulation of ethanol and P_i in the extracellular environment (Table 3). Both these factors have previously been reported to influence nutrient uptake kinetics; an interaction between ethanol and transport proteins have been postulated [33]. 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 [23].

The biomass yield on glucose decreased with increasing dilution rates (Table 3). Changes of the ethanol concentrations and ethanol yield coefficient with changing dilution rates are shown in Table 3 and Fig. 4, respectively. The ethanol yield coefficient increased to a value of 0.24 at dilution rate of 0.22 h^{-1} and remained constant at this value over the dilution range of 0.22-0.45 h^{-1}. Altogether, these results appear to indicate an increase in the fermentative component of the metabolism. The increased fermentative activity of the yeast cells with increasing dilution rate, indicated by the increase in the rate of ethanol production and yield and decrease in the biomass yield coefficients is also expected to affect the growth kinetics.

Kinetic characterization of P_i membrane transport in a continuous culture

In vivo studies of nutrient transport in general have the inherent problem of the interference of subsequent

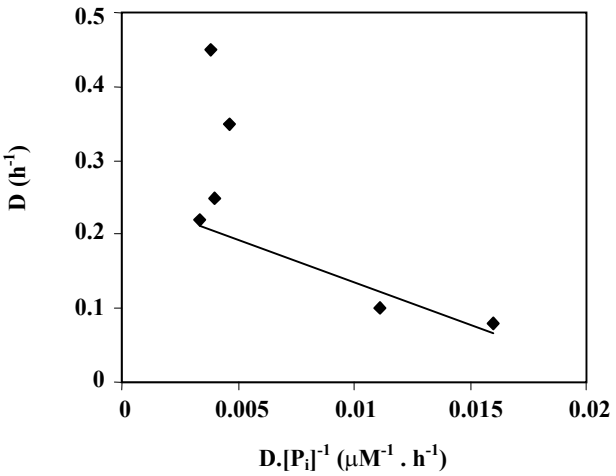

Fig. 3: Eadie-Hofstee plot of the residual P_i concentration as a function of dilution rate.

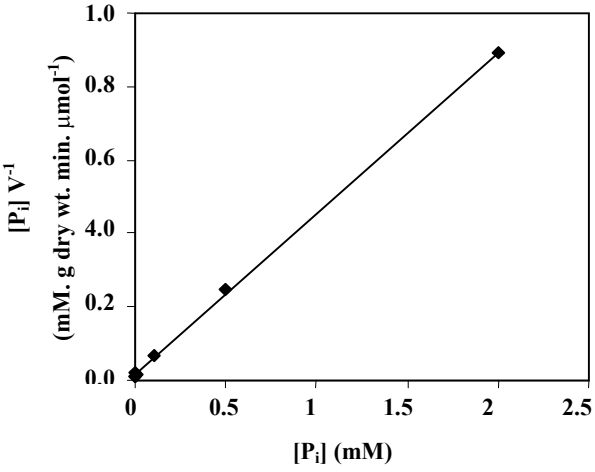

Fig. 5: Hanes-Woolf plot for P_i transport by S. cerevisiae cells from a P_i-limited culture growing at D=0.45 h^{-1}.

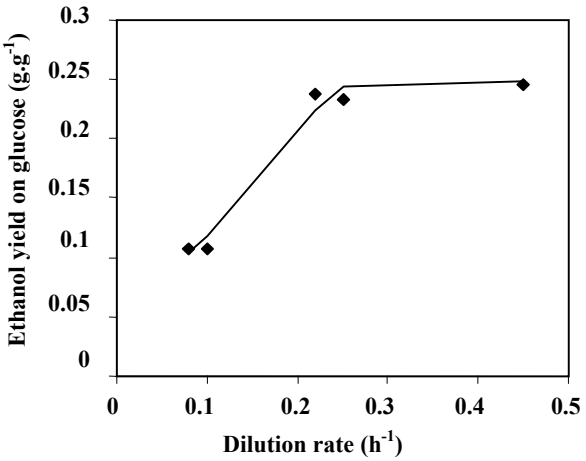

Fig. 4: Ethanol production yield coefficient of S. cerevisiae cells grown in a chemostat culture under various glucose concentrations caused at different dilution rates.

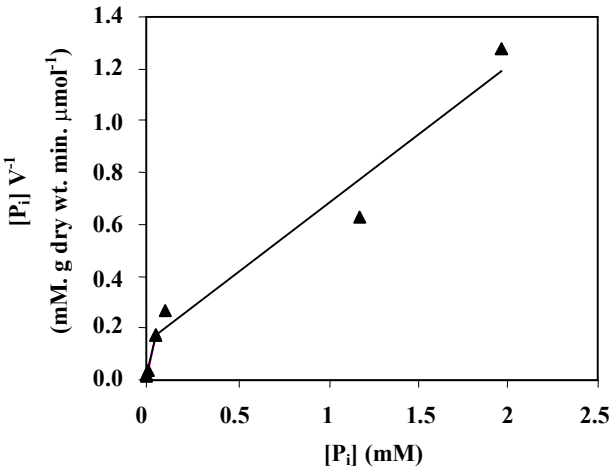

Fig. 6: Hanes-Woolf plot for P_i transport by S. cerevisiae cells from a P_i-limited culture growing at D=0.22 h^{-1}.

metabolism of the substrate. A common method, therefore, for measuring kinetic parameters in nutrient transport is the *zero-trans* method [5,21,37,45,47,48]. By this method, the initial specific rate of P_i uptake rate into cells is measured by ^{32}[P] accumulations. Measurements of the initial rate of P_i uptake as a function of exogenous P_i concentrations provide an estimate of the affinity constant (K_m) and maximum rate (V_m) of P_i uptake. In the present study, P_i concentrations in the range 0.4-2000 μM were used for ^{32}[P] uptake measurements. This wide range of P_i concentrations appropriately covers the different affinity constants of the different P_i transporters of *S. cerevisiae*.

The relationship between P_i uptake rates of cells grown at all dilution rates tested and extracellular P_i concentrations showed good agreement with previous observations in which P_i transport *via* membrane transporters followed Michaelis-Menten kinetics [8,13, 14,49]. At applied dilution rates of 0.1, 0.35, and 0.45 h^{-1} a single component transport system is indicated (Fig. 5). In contrast, however, at a dilution rate of 0.22 h^{-1} the presence of two kinetically different transport systems was indicated (Fig. 6). The apparent affinity constants (K_m) and transport capacity (V_m) calculated from the kinetic data are given in Table 4.

The kinetics seen at D=0.22 h^{-1}, which corresponds to

Table 4: Affinity constants and transport capacities of the P_i transport estimated from Hanes-Woolf plots of P_i uptake by cells grown at different dilution rates in P_i-limited chemostat cultures.

D (h^{-1})	P_i conc. (μM)	K_{m1} (μM)	V_{m1} (μmol.g dry wt^{-1}. min^{-1})	R^{2*}	K_{m2} (μM)	V_{m2} (μmol.g dry wt^{-1}.min^{-1})	R^{2*}
0.1	9	6.8	4.83	0.99	–	–	–
0.22	65	1.7	0.30	0.99	284	1.92	0.96
0.35	76	9.9	1.31	0.99	–	–	–
0.45	117	36	2.28	0.99	–	–	–

** Correlation coefficients of linear regression*

P_i concentration of 65 μM, indicate the presence of two active P_i transport systems, a high-affinity system with a K_{m1}=1.7 μM and a medium/low affinity system with a K_{m2}=284 μM. The transport capacity of the latter system is 6.4-fold higher than that of the high-affinity transporter (V_m=1.92 vs. 0.3 μmol. g dry wt^{-1}. min^{-1}) at this growth condition. The observed high K_m value (i.e. 284 μM) is close to the K_m values of 216, 205 and 181 μM proposed for the low-affinity P_i transporters of *S. cerevisiae*, Pho87p, Pho90p and Pho91p, respectively [14]. However, these reported K_m values were measured under conditions when the individual P_i transporters were over expressed in a batch culture under high-P_i conditions in a null background strain lacking the other transporters. The individual contribution of the Pho87, Pho90 and Pho91 protein in P_i transport and their physiological regulation is so far unknown. It is likely that the observed apparent kinetics of a low affinity system is due to affinity differences and kinetic mechanisms appearing when cells are transferred from low to intermediate P_i conditions.

Samples taken from the chemostat at D=0.1, 0.35 and 0.45 h^{-1} and analyzed for P_i uptake kinetics did not reveal a K_m characteristic of a low affinity system. Under these conditions the transport system monitored is that of high affinity uptake of P_i. The calculated K_m values (1.7-36 μM) clearly indicate the presence of a high-affinity P_i transport activity at all dilution rates tested. The K_m values reflecting a high affinity uptake is lower than the value of 45 μM reported by Lagerstedt et al. [37] for the Pho84 high-affinity transporter of *S. cerevisiae* CEN.PK113-5D cells grown in YPD medium by a batch cultivation technique. A small contribution of low-affinity transport in P_i uptake has been previously reported using cells, in which both high-affinity transporters (Pho84p and Pho89p) were deleted, grown at low-P_i concentration [6,8].

The transport kinetic data obtained at D=0.45 h^{-1} (corresponding to an external P_i concentration of 117 μM) shows that the high-affinity transport system was expressed at P_i concentrations which according to findings of previous shake flask studies are repressible P_i concentrations [5,9]. In addition, as pointed out before there are some other phenomena which are observed in the present study but not seen in the previously reported batch studies [9,5,8,37,49]. These include: occurrence of low affinity transport system at P_i concentration as low as of 65 μM (D=0.22 h^{-1}) and the expression of high affinity transport system at P_i concentrations higher than 100 μM (at D=0.45 h^{-1}). These observations could be the result of the difference in the strain, media (defined instead of complex) and/or cultivation technique (chemostat instead of batch) used in this study compared to previous studies.

The capacity (V_m) of the high-affinity P_i transport system was at its maximum (4.83 μmol. g dry wt^{-1}. min^{-1}) when a low dilution rate (D=0.1 h^{-1}) was applied, and at its minimum (0.3 μmol. g dry wt^{-1}. min^{-1}) at the dilution rate of 0.22 h^{-1}. Higher dilution rates (0.35 and 0.45 h^{-1}) conferred a slight increase in V_m (Table 4) suggesting the contribution, but not necessarily the dominance, of low-affinity components of P_i transport.

The observed parallel phenomena; i.e. changes in kinetics of phosphate transport, deviation of cells' growth from Monod kinetic model and increase in fermentative activity of the yeast cells; happened at dilution rate around 0.2 h^{-1} probably shows some changes in metabolism of *S. cerevisiae* at this dilution rate.

Acknowledgements

The authors are grateful for financial support from the KK Foundation, Human Frontier Science Organization, and Växjö University, Sweden. S. Sh would like to thank Ministry of Science, Research and Technology of Iran for providing a scholarship during her residence in Sweden.

Received : 28th June 2004 ; Accepted : 18th October 2004

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