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Vertebrate Cell Culture II and Enzyme Technology

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Federico Parisi † 1989

The Advances in Biochemical Engineering/Biotechnology has received with deep sadness the message of the sudden death of Prof. Federico Parisi who leaves behind many friends and colleagues in many countries. A vast scientific community of biotechnology throughout Europe and other continents is mourning over the loss of our Italia pioneer in biotechnology.

Federico Parisi left us in the middle of an active life. He was the promoter of Italy as a candidate for hosting the forthcoming Congress of Biotechnology in 1993 and acting President of its organizing committee.

We met Federico Parisi first twenty years ago at an IUPAC Commission Meeting representing Italy. His broad expertise was recognized by all members of that body when he proposed the elaboration of the alcohol tables. Under his guidance they were later published by the International Union of Pure and Applied

Chemistry and are still in use worldwide. The work has been a great example of international collaboration and efficient committee work by experts. Federico Parisi supported the commission work constantly and represented his very beloved country at the IUPAC-Symposium many times.

Federico Parisi was a particular good friend of Switzerland where he received part of his scientific education under Prof. de Diesbach at Freiburg University. Born in Naples, he graduated in Industrial Chemistry at the University of Milan and completed his professional education at the Laboratory of Research of the Institute de Angeli (Milan) and the Laboratory Sauter (Geneva) before entering the Research Labs of Distillation, a group of ERIDANIA in 1948.

As a leader of this group from 1957 he developed an active scientific life in applied research and academic teaching at the University of Genoa. He became the prominent member of the Italia biotechnology community. As a Professor of the Genoa University he developed very fruitful activities including publication of several most valuable books and editing the official magazine of the Italien Chemical Society "La Chimica e l'Industria" (from 1970 to 1985). A most relevant review article on "Advances in Lignocellulosics Hydrolysis and the Utilization of the Hydrolyzates" has been published in the last volume of this series (Vol. 38, 1989) informing us of his great interest in bioenergetics.

During the preparatory period for founding the European Federation of Biotechnology (EFB) he promoted the founders efficiently and he was elected as the first representative of his country in the Executive Committee of EFB.

Besides his scientific and professional qualities Federico Parisi impressed all of us by his humanity and great enthusiasm. We remember him as a perfect gentleman and a sincere friend.

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Metabolic Control of Glucose Degradation in Yeast and Tumor Cells

Armin Fiechter and Felix K. Gmünder
Institute of Biotechnology, ETH-Hönggerberg, CH-8093 Zurich, Switzerland

Regulation of glucose degradation in both yeasts and tumor cells is very similar in many respects. In both cases it leads to excretion of intermediary metabolites (e.g. ethanol, lactate) in those cell types where uptake of glucose is unrestricted (*Saccharomyces cerevisiae*, Bowes melanoma cells).

The similarities between glucose metabolism observed in yeast and tumor cells is explained by the fact that cell transformation of animal cells leads to inadequate expression of (proto-)oncogenes, which force the cell to enter the cell cycle. These events are accompanied by alterations at the signal transduction level, a marked increase of glucose transporter synthesis, enhancement of glycolytic key enzyme activities, and slightly reduced respiration of the tumor cell. In relation to homologous glucose degradation found in yeast and tumor cells there exist strong similarities on the level of of cell division cycle genes, signal transduction and regulation of glycolytic key enzymes.

It has been demonstrated that ethanol and lactate excretion in yeast and tumor cells, respectively, result from an overflow reaction at the point of pyruvate that is due to a carbon flux exceeding the capacity of oxidative breakdown. Therefore, the respiratory capacity of a cell determines the amount of glycolytic breakdown products if ample glucose is available. This restricted flux is also referred to as the respiratory bottleneck. The expression "catabolite repression", which is often used in textbooks to explain ethanol and acid excretion, should be abandoned, unless specific mechanisms can be demonstrated.

Furthermore, it was shown that maximum respiration and growth rates are only obtained under optimum culture conditions, where the carbon source is limiting.

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1 Introduction

Sugars have a profound influence on the metabolism of man and microbes. They provide the main source of carbon and energy for cell physiology and proliferation. Among the mono- and polysaccharides, glucose plays a central role in the living world due to its widespread utilization. It is the substrate par excellence and represents the starting point of the major metabolic pathways.

Control mechanisms of glucose degradation have therefore been the focus of research since Pasteur [86] observed reduced ethanol formation in yeast in the presence of oxygen ("Pasteur-effect"). Furthermore, dominance of glucose uptake over other sugars and formation of certain metabolites became known as the "glucose effect". This phenomenon is also known as "catabolite repression", which refers to first, the inhibition of degradation of other substrates by the breakdown products of glucose, and second, reduced respiration when glucose serves as the carbon source. However, Fiechter et al. [32] have shown that the control of metabolic pathways underlying this "glucose effect" occurring in glucose sensitive yeasts has never been elucidated. It is clear that "fermentation" (or excretion of ethanol) by yeasts or formation of lactate by animal cells was related to glucose and oxygen consumption rates or fluxes. Thus, it was realized that the control of respiration became a key aspect in the control of glycolysis.

In the past, regulation of glucose metabolism was studied on the level of substrate or metabolite concentrations instead in terms of turnover rates e.g. metabolite kinetics. Therefore, methodological concepts remained inadequate for decades and even the introduction of "defined" systems using respirometer measurements with resting cells turned out to be artifactual with respect to the in vivo situation of growing cells.

In more recent years, progress has been made in studying the regulation of glucose metabolism due to the improvement of cultivation methods for microbial and animal cells. Growing cell populations were investigated instead of resting cells using chemostat methods. The use of chemostat experimentation including pulse and shift technique has led to new insights into the coordination of metabolic activity as in these cases the growth rate and culture conditions are well defined. Thus, reconsideration of old problems like "catabolite repression" during glucose degradation or regulation of respiration during growth have become more promising. Progress made in animal cell culture in particular will allow a better comparison of glucose metabolism to be made between higher and lower eukaryotes.

Recently, the regulation of glucose metabolism in *Saccharomyces*-type yeasts has been studied using feed-controlled systems [54, 55, 112] as well as ^{13}C NMR and ^{14}C radioactive labelling techniques in accordance with the Warburg manometric methods. In particular, its level of aerobic and anaerobic glycolysis were determined, and the kinetics of yeast phosphofructo 1-kinase (PFK-1) was studied in vitro [25, 26, 97]. More recently, our knowledge of regulation of glycolysis in yeast has been widened by studying the involvement of fructose 2,6-biphosphate in this process [19, 121]. Today, all data available on this metabolic pathway firmly suggest that its regulation is closely linked with the cell's respiration capacity. Thus, under condition of excess glucose uptake rates, the respiratory capacity of the cell is exceeded, and ethanol formation begins [54, 55, 112]. This observation led to the formulation of a model, which explains

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this overflow of glycolysis products at the point of pyruvate, by the respiratory bottleneck [55].

In contrast to yeast cells, much less is known regarding the control of glycolysis in tumor cells. Most of these cells consume considerably more glucose than their normal ancestors do. This increased glycolytic activity of tumor cells is a well established phenomenon since the time of Warburg [127]. Moreover, they secrete large amounts of lactate, even in oxygen saturated media, where respiration should be optimal.

This increase of aerobic glycolytic activity in tumor cells may presently be explained by, first, an increased number of glucose transporters [10, 34], second, an increased level of mitochondrial hexokinase (HK) isoenzyme [29, 135], and third, a decrease in the number of mitochondria in tumor cells, leading to ineffective oxidation [29, 87].

A comparison of the metabolic control of glycolysis in yeast and tumor cells allow us to present an updated view of this process.

2 Historical Perspectives

Pasteur studied "fermentation" and the formation of ethanol by yeast. A full understanding of regulation of glycolysis, respiration and growth, however, used to be a subject of confusion and controversy as Pasteur did not measure glucose uptake or growth rates. Instead, he estimated ethanol formation using ill defined systems containing growing cells and excessive sugar. Pasteur reported in 1861 [86] that the efficiency of "fermentation" decreases in the presence of air. This observation was termed the "Pasteur effect" by Warburg [129] in relation to his studies on glucose metabolism in tumors using his newly developed manometric method. Warburg determined lactate and carbon dioxide production in carbohydrate-utilizing cells and tissues. Concentrations of the end products of glycolysis suggested that tumor cells possess a high glycolytic activity and low respiration in comparison with normal cells [127]. Warburg concluded that low respiration is the origin of cancer [127, 129]. This startling yet false conclusion was maintained by the Warburg-school for more than 30 years and led to endless controversies between supporters and opponents [15, 92, 93, 130, 131, 133]. High glycolysis and low respiration is also observed in a variety of normal proliferating and resting cells that have either few or no mitochondria [29].

In contrast to Warburg, Crabtree noticed a slight reduction (10%) of the respiration in various tumor cells [22]. Crabtree concluded that in spite of a relatively high respiration, this is ineffective in reducing the aerobic glycolysis. He also noted that the glycolytic activity of tumors exerts a regulatory effect on their respiration. However, Crabtree observed that respiration and carbohydrate metabolism varied widely between tumor tissue of different origin. Nevertheless, increased glycolysis was always observed in tumor cells [87, 133]. Thus, Crabtree stated that respiration is unable to affect the glycolytic rate. This is in contrast to Pasteur's observations, which show that respiration does influence the rate of glycolysis.

3 Regulation of Glycolysis

3.1 Yeast

Glucose appears to be the predominant sugar involved in regulating glycolysis and respiration. In the presence of glucose many strains of microbes typically show reduced cell synthesis (biomass yield), respiration, activity of enzymes and excretion of metabolites derived from pyruvate. If glucose and other carbohydrate sources are used simultaneously as a carbon and energy source, glucose is always preferentially degraded. Thus, any model has to explain the interrelationship between glucose uptake, glycolysis and respiration. Taken together, one has to deal with a rather intricate situation which cannot be explained just by the current textbook explanation of "catabolite repression".

3.1.1 Early Experimental Data

The first major hypothesis for regulation of glycolysis in yeast was proposed by Johnson [51] and Lynen [71] in 1941. Johnson regarded reduced glycolysis, as observed by Pasteur, as being due to decreased concentrations of inorganic phosphate (P_i), AMP and ADP. Lynen [71] proposed that glycolysis and oxidative phosphorylation compete for P_i. In the presence of air respiration succeeds and reduces the availability of P_i for generation of ATP at the substrate level. This idea of a key role of P_i in regulating carbohydrate metabolism was further supported by investigations where the Pasteur effect was eliminated by uncoupling the respiratory chain and oxidative phosphorylation [72]. It was believed that this leads to increased levels of P_i, AMP and ADP that in turn favors high rate of glycolysis. This view is further supported by the observation that glucose uptake and ethanol excretion were augmented.

In the following 10 years, Lynen discovered that under aerobic conditions, where glycolysis is reduced but respiration is increased, the activity of hexokinase (HK) is low. Since the activity of HK is ATP dependent, one would expect the activity of HK to be high when respiration is increased. Lynen and Koenigsberger [70] suggested that the slowing-down of the HK-reaction might be due to feedback-inhibition of glucose phosphorylation by glucose 6-phosphate or to a decrease in ATP-concentration in the cytosol because it is compartmentalized within the mitochondria. In this case, the HK-reaction would be impaired due to lack of ATP at the site of glucose phosphorylation [69].

In addition to the above regulators of glycolysis it became clear that other effectors and mechanisms of regulation must exist. For instance, Chance [17] showed that in isolated mitochondria respiration and oxidative phosphorylation is regulated by the concentration of ADP. Shortly thereafter the concept of allosteric regulation was established [92, 93]. Allosteric enzymes are regulatory enzymes, which are inhibited or stimulated by end-products of the metabolic sequence (negative or positive feedback, respectively). The inhibitory or stimulating metabolite is known as the effector. The allosteric regulation of glycolysis in yeast was summarized in a model by Sols et al. [111] in 1971. According to this, regulation of glycolysis is based on negative or positive feedback regulation by PFK-1. A decrease of PFK-1 activity is observed when citrate and ATP concentrations increase under conditions where respiratory activity is high

and glycolysis is reduced. In contrast, an increase in PFK-1 activity occurs when concentrations of ammonium ions, ADP and AMP are high, under conditions of restricted respiration and increased glycolysis. This model could account for the "Pasteur effect", namely the inhibition of glycolysis by respiration, and the "Crabtree" or "glucose effect", namely the inhibition of respiration if ample glucose is available.

3.1.2 Current Concepts

As opposed to earlier studies, more recent investigations used growing cells for the dynamic analysis of glucose and oxygen turnover rates [54, 55, 112]. Now, two different modes of glucose metabolism in yeast are described in relation to control of glucose uptake.

In obligate respirative yeast, such as *Trichosporon cutaneum*, where the specific glucose uptake rate q_S is strongly correlated to the specific oxygen uptake rate q_{O_2} (Table 1, Fig. 1). Thus, if the respiration rate is low, glucose uptake is low; in addition no ethanol is excreted, and if glucose is supplied in excess it accumulates extracellularily. The reason for this behavior is not clear. Obligate respirative fungi, such as *Chaetomium cellulolyticum*, exhibit the same growth characteristics (Fig. 2).

In contrast, in glucose sensitive yeast (facultative anaerobic), such as brewer's and baker's yeast, glucose uptake (q_S) is not controlled by the respiration rate (q_{O_2}). In these strains when sugar is supplied in excess, surplus glucose is excreted as ethanol (Table 1, Fig. 3).

Candida tropicalis represents an intermediate form between the above. When oxygen is not limiting and glucose is in excess, glucose uptake is controlled, therefore no ethan-

Table 1. Typical data of growth parameters of glucose sensitive and insensitive yeast strains. Defined media and carbon limitations were used in the chemostat. It is noteworthy that the highest glucose uptake rates are found in glucose sensitive strains and not in strict oxidative types. *Candida tropicalis* is an intermediate form excreting ethanol under conditions of O_2-limitation (for details s. [2, 49, 68, 100, 104]). For abbreviations s. p 24

Cell types		Effectors	D_c h^{-1}	$Y_{X/S}$ —	q_{O_2} mmol g^{-1} h^{-1}	$q_{S_{max}}$ mmol g^{-1} h^{-1}
I	*Trichosporon cutaneum* (obligate aerobis)	O_2	0.4	0.50–0.6	8	≅5
II	*Candida tropicalis* (obligate aerobic)	O_2, (C_6)	0.6	>0.5	14	<20
III	*Saccharomyces cerevisiae* (aerobic: diauxic growth)	O_2, C_6	0.4	0.5	8	20
IV	*Saccharomyces cerevisiae* (anaerobic: monoauxic growth)	C_6	0.3	0.1	—	20
V	*Schizosaccharomyces pombe* (monoauxic growth)	O_2, C_6	0.35	0.3	1.5	≅5
VI	*Chaetomium cellulolyticum* (monoauxic growth)	O_2	0.29	0.51	7.62	0.6
VII	*Bowes melanoma cells* (diauxic growth)	C_6	0.02	0.15	0.24 [h^{-1}]	0.15 [h^{-1}]

ol is produced. In contrast, under conditions of oxygen-limitation, *C. tropicalis* excretes ethanol (Table 1, Fig. 4).

In *Schizosaccharomyces pombe* glucose uptake is not controlled. Therefore it forms ethanol. In this particular yeast ethanol cannot be used for synthesis of biomass during

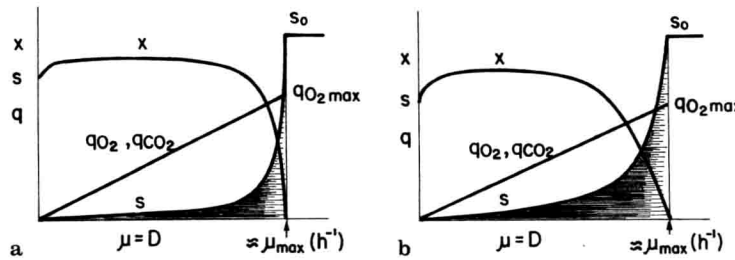

Fig. 1 a and b. *Trichosporon cutaneum* is a glucose insensitive yeast strain that is not affected by glucose or oxygen in continuous cultivation (**a**, glucose limitation; **b**, oxygen or trace element limitation; [49, 50, 59, 60]). Under the condition of oxygen or trace element limitation (Fe or Mo; **b**), the maximum specific oxygen uptake rate begins to decrease at lower dilution rates as compared to carbon limitation (**a**).

In the case of oxygen or trace element limitation D_C is reduced by about 10%. For typical values s. Table 1; for abbreviations s. p 24. The chemostat diagrams indicate that a strong glucose uptake control exists, which is strictly correlated to the respiration. As a consequence, residual glucose appears in the medium at lower dilution rates (represented by the dashed area under the S-graph) as compared to baker's yeast (s. Fig. 3). The (hitherto unknown) control mechanisms of glucose uptake by respiration prevents an overflow reaction at the point of pyruvate and no ethanol excretion appears under any condition. The activity of the glycolytic key enzymes, which are hexokinase, phosphofructo 1-kinase and pyruvate kinase, may be controlled by effectors such as ATP, fructose 1,6-biphosphate and citrate (s. also Fig. 13)

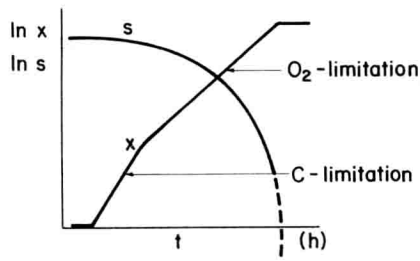

Fig. 2. Batch growth of *Chaetomium cellulolyticum* (obligate aerobic) exhibits a strict monoauxic growth pattern in batch cultures that is familiar among molds [125]. It is a glucose (and oxygen) insensitive cell type. The specific growth rate μ reaches its maximum during a first growth period when growth is carbon limited. The growth rate decreases when oxygen becomes the limiting factor, however, no ethanol is excreted. This behavior is typical for many strict aerobic fungi, where glucose uptake seems to be controlled by the respiration rate. Maximal growth rate μ_{max} of *Chaetomium cellulolyticum* is rather small, as compared to yeasts, but yield $Y_{X/S}$ and RQ similar. For typical values s. Table 1; for abbreviations s. p 24. It is noteworthy that no reliable data from chemostat experiments are available — this is due to experimental difficulties. Glucose uptake of this type of fungi is similar to *Trichosporon*, i.e. it is strongly associated with the respiratory capacity. Therefore, an overflow reaction and excretion of metabolites (ethanol, lactate, acid) does not occur

a second growth phase in batch cultures since it lacks gluconeogenetic capability (missing glyoxylic pathway). Nevertheless, ethanol is oxidized completely after glucose consumption resulting in a secondary monoauxie in batch cultivation (Table 1, Fig. 5).

Studies of *Saccharomyces cerevisiae* in continuous cultures showed that the specific oxygen uptake rate q_{O_2} is proportional to the dilution rate up to the point D_R when ethanol production begins and the oxygen uptake rate remains constant [100]. Thus, at high dilution rates above D_R a dramatic shift from a purely oxidative glucose breakdown to a mixed form of glucose degradation was noted. This observation was the starting point for the formulation of a new regulatory concept [112], in which ethanol

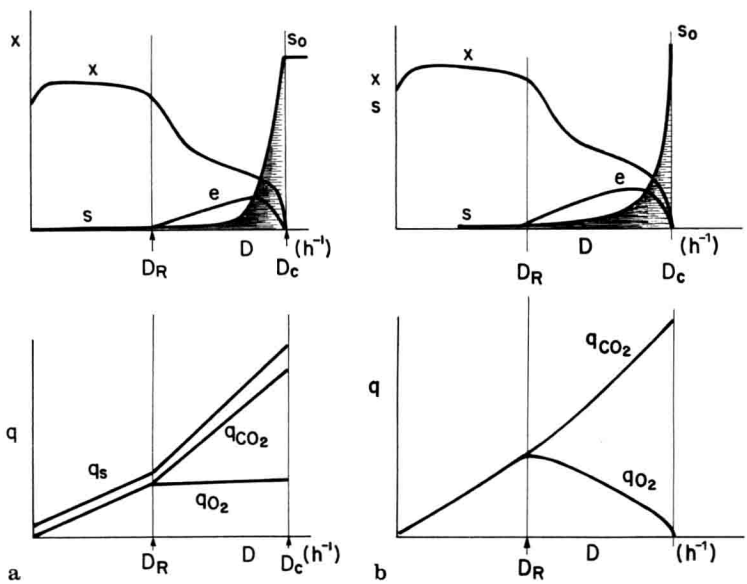

Fig. 3a and b. The growth behavior of *Saccharomyces cerevisiae* is typical for the so called glucose sensitive yeasts, in which glucose uptake is not controlled by the respiration rate. In glucose sensitive yeast strains surplus glucose is excreted as ethanol. Some bacteria, such as *Escherichia coli* exhibit a similar growth pattern leading to acetate excretion [78, 98]. Similarly, in transformed animal and human cell lines glucose uptake is increased as compared to normal cells, leading to lactate excretion [65, 66]. **a** Chemostat culture of *S. cerevisiae*; under the condition of carbon limitation. The specific oxygen uptake rate q_{O_2} is proportional to the dilution rate up to the point D_R, when ethanol excretion begins and the oxygen uptake remains constant until washout. **b** When oxygen or trace elements are limiting, D_R is markedly reduced. For typical values s. Table 1; for abbreviations s. p 24. The dashed area under the S-graph indicates that no residual glucose is present in the medium when the dilution rate is below D_R. In other words uptake is not under control of the respiration rate. This results in the excretion of ethanol since the glycolytic flux exceeds the capacity of the respiratory pathway. The area under the S-graph in **b** is larger than the corresponding area in **a** — this is due to restricted respiration. In this case the respiration is restricted by a limitation in O_2, Fe, Mo, etc. In relation to this, compare also **1a** and **1b**. A new regulatory concept was described recently, which explains ethanol formation by glucose sensitive yeasts as an overflow reaction of the glycolytic pathway when the respiratory capacity is exceeded [54, 55, 112]

formation is explained as an overflow reaction of the glycolytic pathway when the capacity of the TCA-cycle and mitochondrial respiration is exceeded. A schematic representation of the model and its variables is given in Fig. 6. The model is flexible enough to meet all variants of genera- and species-specific regulatory concepts of glucose breakdown.

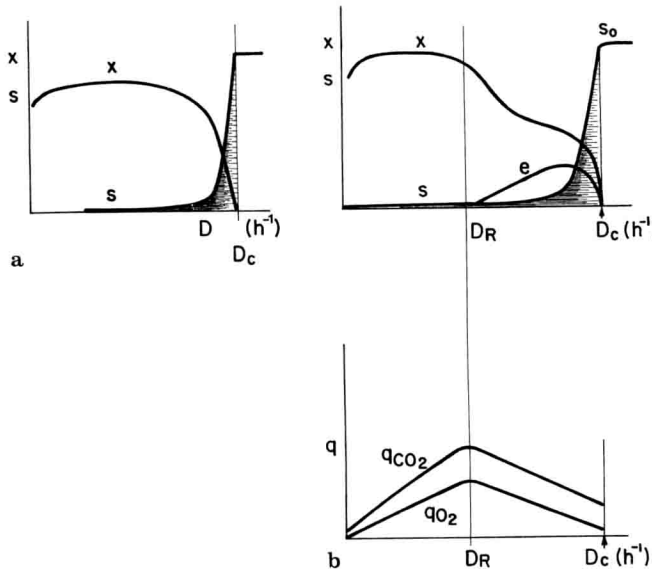

Fig. 4a and b. In *Candida tropicalis* glucose uptake and breakdown is controlled by the rate of oxygen uptake under the condition of carbon limitation (**a**). Therefore, no ethanol is produced (monoauxic growth in batch culture). In contrast, under conditions of oxygen limitation (**b**) this yeast excretes some ethanol (diauxic growth in batch culture). In continuous cultivation *Candida tropicalis* shows similar growth characteristics as *Trichosporon* and related fungi when growth is carbon-limited. Because *C. tropicalis* has a high respiratory capacity, glucose is completely oxidized until washout. The RQ remains constant at all dilution rates. For typical values s. Table 1; for abbreviations s. p 24. However, under conditions of oxygen limitation, ethanol is produced above a dilution rate $D_R \cong 0.3$ (which depends on the degree of limitation) that is similar to growth characteristics of *Saccharomyces*-type yeast (s. Fig. 3)

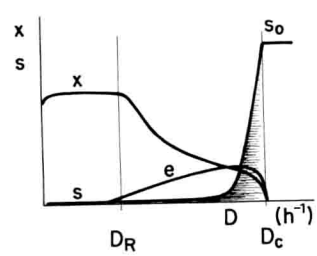

Fig. 5. In the fission yeast *Schizosaccharomyces pombe* glucose uptake is not controlled by respiration, thus ethanol is excreted (carbon-limitation). In addition, it is very likely that this yeast is also oxygen sensitive. *S. pombe* has an unusual low yield both in batch and continuous cultivation, and maximum growth rates (μ_{max}) are rather small. For typical values s. Table 1; for abbreviations and symbols s. p 24

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