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Growth of *Schizosaccharomyces pombe* on Glucose-Malate Mixtures in Continuous Cell-Recycle Cultures

Kinetics of Substrate Utilization

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ABSTRACT

The aerobic growth of *Schizosaccharomyces pombe* on mixtures of glucose and malate was investigated during continuous high cell density cultures with partial cell-recycle using a membrane bioreactor. Determination of the specific metabolic rates relative to substrates and products allowed the capacity of the yeast to metabolize malic acid under both oxidative metabolism (carbon limited cultures) and oxidofermentative metabolism (carbon sufficient cultures) situations to be characterized. Under carbon limiting conditions, the specific rate of malate utilization was dependent on the residual concentration and a limit for a purely oxidative breakdown without ethanol formation was observed for a characteristic ratio between the rates of substrate consumption q_M/q_G of 1.63 $g.g^{-1}$. In addition, the mass balance analysis revealed the incorporation of malic acid into biomass. In carbon excess environments, the specific rate of malate utilization was dependent on both the residual malate and the specific rate of glucose consumption indicating that in addition to its conversion into ethanol malate can be respiratively metabolized for q_M/q_G ratios higher than 0.4 $g.g^{-1}$.

Index Entries: *Schizosaccharomyces pombe*; malate utilization; growth kinetics; cell-recycle culture.

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INTRODUCTION

Lactic acid bacteria are generally used to remove malic acid from musts and wines. However, because of some technical difficulties in the control of their growth, the potential use of yeasts of the genus *Schizosaccharomyces* as starter cultures has often been suggested *(1–4)* and promising results obtained for grape must deacidification *(5,6)*.

Since the physiology of the fission yeast *Schizosaccharomyces pombe* is similar to that of *Saccharomyces cerevisiae* (glucose sensitive yeasts), most of the studies have concerned the maloalcooholic fermentation pathway *(7)* and the malate transport systems *(8,9)*. Only few investigations have been undertaken concerning the kinetic aspects of growth and malate conversion *(10)* and, because of lack of accurate quantification of the metabolic rates during growth on glucose alone and glucose-malate mixtures, the anabolic role of malic acid has never been clearly established.

In a previous paper the effects of various operating conditions on both the growth kinetics of *Schizosaccharomyces pombe* on glucose and metabolic status of the cells during high cell density cultivation with total cell retention were reported *(11)*. From further experiments, carbon and energy balances were analyzed during steady-state culture regimes obtained at various specific growth rates *(12)*, and the energetics of growth under glucose limited and glucose excess environments were examined and discussed *(13)*.

In this article the aerobic growth of *Schizosaccharomyces pombe* on mixtures of glucose and malic acid in continuous high cell density cultures with partial cell recycle was investigated. A kinetic analysis was carried out on the basis of the specific metabolic rates with a view to obtain precise information on the capacity of the yeast to metabolize malic acid under both carbon limited (oxidative metabolism) and carbon excess (respirofermentative metabolism) environments.

MATERIALS AND METHODS

Organism and Culture Medium

The strain of *S. pombe* was supplied by the Institut Cooperatif du Vin (Montpellier, France). It was maintained on a medium containing: glucose 20 g/L; yeast extract 5 g/L; asparagine 4 g/L; KH_2PO_45 g/L; $MgSO_4$ 0.4 g/L; Agar 20 g/L. The synthetic medium, the composition of which was representative of a standard grape must, was that proposed by Sablayrolles and Barre *(14)* with glucose at a 50 g/L concentration as the carbon source. Precultures were propagated in Erlenmeyer flasks on the synthetic medium by incubation at 30°C on an orbital shaker.

Analytical Methods

Biomass concentration was measured turbidimetrically at 640 nm (Kontron Model Unikon 810 spectrophotometer), after dilution of the sample when necessary, and calibrated to cell dry weight determinations. For dry weight measurements, cells were harvested by centrifugation in Eppendorf tubes, washed twice with distilled water, and dried to constant weight at 60°C and under partial vacuum.

Residual glucose concentration was measured in the permeate using an YSI glucose analyzer (Yellow Springs, OH). Malic acid, pyruvic acid, glycerol, and acetaldehyde concentrations were determined by HPLC using an ion-exclusion column Ion 300 (Interaction Chemical, Mountain View, CA) using a refractive index detector with 0.025*N* sulphuric acid as eluent (flow rate 0.5 mL/min). Ethanol concentration was measured by gas-liquid chromatography (FID detector) on a Porapak Q column (temperature 190°C; nitrogen flow rate 30 mL/min) with isopropanol as internal standard.

The outflow gas rate was measured volumetrically with a wet gas meter, and carbon dioxide production and oxygen consumption were determined by measuring their concentrations in the outflow gas using a gas chromatograph (column of Porapak Q + molecular sieve 5 Å, 80–100 mesh; temperature 40°C; helium flow rate 20 mL/min). The dissolved oxygen concentration was measured with an Ingold probe and monitored by coupling to an oxygen-meter.

The elemental composition of biomass was determined as described elsewere *(13)*.

Process Equipment, Procedures, and Culture Conditions

The cell-recycle fermentor was based on the fermentation vessel and control instrumentation of a conventional chemostat fermentor. A line-diagramm has been presented in a previous paper *(15)*. The process equipment, including the separation device procedures and operating culture conditions, have been previously described *(12,13)*. The dissolved oxygen concentration was set to around 20% of the air saturation. In addition, the fresh medium was supplied to the culture in concentrated and separated forms, salts + vitamins, glucose, malic acid, using peristaltic pumps with selected feed-flow rates. Finally, the cultures were considered at steady state when no variations in the concentrations of biomass, substrates and products were observed, generally after at least five residence times.

RESULTS AND DISCUSSION

Aerated cultures with partial cell recycle were performed on various glucose-malate mixtures at biomass bleeding rates close to 0.06 and 0.12 h^{-1}, equivalent to specific growth rates. According to the operating conditions,

Table 1
Carbon Limited Cultures (oxidative metabolism) on Various Glucose-Malate Mixtures at Biomass Bleeding Rates (Equivalent to Specific Growth Rates) Close to 0.06 and 12 h^{-1} [a]

A

Dilution rates (h^{-1})		Inlet fluxes ($g.l^{-1}.h^{-1}$)		Concentrations ($g.l^{-1}$)				R.Q.
Biomass (d)	Global (D)	Glucose	Malate	G	M	X	E	(mol.mole^{-1})
0.057	0.234	2.65	0	0.16	0	19.5	0	1.08
0.061	0.234	2.41	0.87	0.17	0.14	20.5	0	1.17
0.056	0.234	1.60	1.56	0.17	0.39	18.9	0	1.28
0.056	0.234	1.20	3.27	0.05	0.52	21.5	0.20	1.45
0.056	0.234	1.20	4.04	0.06	0.75	24.1	1.10	1.72
0.112	0.46	8.40	0	0.13	0	32.7	0	1.10
0.113	0.60	6.10	1.54	0.16	0.16	27.2	0	1.30
0.110	0.53	5.02	3.33	0.13	0.27	30.2	0	1.33
0.113	0.53	4.41	3.80	0.12	0.36	32.0	0	1.40
0.118	0.51	3.36	4.34	0.13	0.46	24.4	0	1.40
0.115	0.45	2.70	4.83	0.13	0.62	23.0	0	1.63
0.116	0.48	1.42	6.20	0.10	1.20	16.5	1.25	1.88
0.110	0.47	0.53	6.20	0.10	4.47	7.1	1.37	2.52

B

Proportion of Malate in the mixture (%, w/w)	d (or μ) (h^{-1})	Specific metabolic rates ($g.g^{-1}.h^{-1}$)					Yield ($gX.mole\ C^{-1}$)
		q_G	q_M	q_O	q_C	q_E	
0	0.057	0.134	-	0.065	0.097	0	12.7
26	0.061	0.115	0.041	0.073	0.117	0	12.1
49	0.056	0.085	0.078	0.072	0.127	0	10.9
73	0.056	0.055	0.147	0.083	0.165	2.2×10^{-3}	9.0
77	0.056	0.050	0.160	0.072	0.170	1.0×10^{-2}	8.7
0	0.112	0.255	-	0.110	0.181	0	13.1
20	0.113	0.221	0.054	0.117	0.200	0	12.6
40	0.110	0.164	0.106	0.098	0.180	0	12.7
46	0.113	0.140	0.113	0.095	0.183	0	13.0
56	0.118	0.136	0.168	0.109	0.210	0	12.3
64	0.115	0.117	0.196	0.194	0.225	0	11.8
81	0.116	0.083	0.340	0.120	0.310	3.7×10^{-2}	8.9
92	0.110	0.065	0.575	0.115	0.450	9×10^{-2}	5.7

[a]A: Concentrations at steady state; B: Specific metabolic rates.
G: glucose; M: Malate; O: Oxygen; C: Carbon dioxide; E: Ethanol.

i.e., varying the relative inlet feed flows of the carbon sources glucose and malate (or glucose alone) and basal medium (salts + vitamins), steady state cultures with high cell concentrations were achieved under separate culture regimes in which carbon was either limiting or in excess.

Growth Characterization on Various Glucose-Malate Mixtures

Biomass, substrate, and product concentrations observed at steady state during carbon limited cultures are given in Table 1A. Progressive substitution of glucose by malate resulted in similar consequences on residual substrate concentrations. Whatever the malate-glucose ratio in the inlet feed flow, the residual glucose in the permeate remained at a similar level, below 0.2 g/L, whereas enhanced residual malate concentrations

were observed as the proportion of malate in the mixture was increased. The respiratory quotient indicated that an oxidative breakdown of substrates occurred in spite of a slight ethanol production under certain experimental conditions. In addition, at all steady state regimes the carbon recovery and the degree of reduction balance where higher than 96%, as no significant difference in the elemental composition was observed for cells grown under the various conditions tested, the previously determined ash-free biomass formula, $CH_{1.9}O_{0.81}N_{0.15}$ was used for calculations *(13)*. The corresponding specific metabolic rates relative to substrates and products are given in Table 1B. At a given μ value, increasing the proportion of malate (given in %, w/w) in the substrate mixtures resulted in enhanced specific rates of malate utilization and a correlated decrease in specific rates of glucose consumption. The specific rates of oxygen consumption and carbon dioxide production remained constant, except when a slight ethanol production occurred, probably as a consequence of a total carbon overflow at the pyruvate level. Under these conditions, a significant decrease in the biomass yield relative to substrates, expressed as gX.mol C^{-1}, was observed.

For cultures performed under carbon excess environments, biomass, substrate, and product concentrations observed during steady state regimes are given in Table 2A. Carbon excess culture conditions resulted in ethanol production and formation of other minor products (acetaldehyde, glycerol, and pyruvate). As observed in carbon limited cultures, malate was never completely exhausted, and enhanced residual concentrations were obtained when the proportion of malate in the inflowing medium was increased. Values of the respiratory quotient showed that growth proceeded with a respiro-fermentative metabolism of the cells. The carbon recovery and the degree of reduction balance were shown to be higher than 98%. For the various culture conditions tested, the specific metabolic rates are presented in Table 2B. No evident conclusion could be directly derived from these values and only an accurate analysis would allow the characterization of the malate utilization to be achieved. It can only be noticed that the yield for biomass on substrate was the expected value for the respiro-fermentative breakdown of glucose by a fermentative yeast *(16)*.

Kinetic Analysis of Malate Utilization

Carbon Limiting Environments

For culture conditions under which a purely oxidative breakdown of substrates without ethanol formation was obtained linear relationships were observed when the experimental yields μ/q_1, were plotted against the ratio between the specific consumption rates of the two substrates, q_M/q_G (Fig. 1). By linear regression analysis the following linear relationships were derived.

Table 2
Carbon Excess Cultures (Oxidoreductive Metabolism) on Various Glucose-Malate Mixtures at Biomass Bleeding Rates (Equivalent to Specific Growth Rates) Close to 0.06 and 0, 12 h-1[a]

A

Dilution rates (h^{-1})		Inlet fluxes ($g.l^{-1}.h^{-1}$)		Concentrations ($g.l^{-1}$)				R.Q.
Biomass (d)	Global (D)	Glucose	Malate	G	M	X	E	(mol.mole^{-1})
0.065	0.395	24.2	0	10.43	0	31.8	20.2	12.6
0.063	0.397	21.57	0.104	5.35	0.138	31.2	19.85	11.4
0.069	0.415	22.46	2.44	7.20	0.352	33.9	18.92	12.1
0.068	0.386	24.23	5.54	9.26	0.95	35.3	23.48	14.9
0.065	0.404	24.10	9.95	12.22	3.12	37.0	25.85	18.5
0.68	0496	26.37	14.50	2.88	4.33	50.4	26.06	12.8
0.071	0509	25.32	15.45	1.58	2.554	54	26.03	11.7
0.119	0.477	31.0	0	4.16	0	25.8	25.67	9.8
0.117	0.473	28.95	1.29	10.40	0.29	24.8	20.44	15.4
0.117	0.472	28.10	2.50	7.80	0.47	22.5	21.10	12.7
0.121	0.479	31.40	5.17	19.90	1.24	21.0	20.57	19.3
0.117	0.479	28.50	8.25	7.70	1.32	27.7	24.86	15.1
0.118	0.478	25.63	8.24	4.15	1.35	28.0	25.43	7.9
0.114	0.475	12.97	18.27	0.35	4.79	25.5	19.32	10.6

B

Proportion of Malate in the mixture (%, w/w)	d (or μ) (h^{-1})	Specific metabolic rates ($g.g^{-1}.h^{-1}$)					Yield ($gX.mole\ C^{-1}$)
		q_G	q_M	q_O	q_C	q_E	
0	0.065	0.630	-	0.013	0.230	0.250	3.10
4	0.063	0.612	0.031	0.018	0.287	0.247	2.95
9.6	0.069	0.575	0.068	0.017	0.286	0.230	3.25
19.2	0.068	0.585	0.147	0.015	0.337	0.257	2.90
30.6	0.065	0.518	0.234	0.012	0.361	0.260	2.70
36.3	0.066	0.495	0.245	0.016	0.363	0.256	2.81
38	0.071	0.454	0.262	0.020	0.362	0.245	3.09
0	0.119	1.125	-	0.033	0.446	0.474	3.17
4.0	0.117	0.993	0.048	0.027	0.463	0.400	3.38
8.1	0.117	1.081	0.101	0.029	0.528	0.442	3.0
14.5	0.121	1.041	0.218	0.020	0.577	0.469	2.94
20.2	0.117	0.894	0.275	0.026	0.559	0.429	3.08
24.6	0.118	0.880	0.271	0.026	0.557	0.434	3.15
58.6	0.114	0.502	0.627	0.044	0.628	0.360	3.22

[a]A: Concentrations at steady state; B: Specific metabolic rates.
G: glucose; M: Malate; O: Oxygen; C: Carbon dioxide; E: Ethanol.

$$\mu = 0.336 q_M + 0.435 q_G \quad (1)$$

$$q_o = 0.302 q_M + 0.467 q_G \quad (2)$$

$$q_C = 0.711 q_M + 0.726 q_G \quad (3)$$

Note that the experimental yield for biomass on glucose, μ/q_G, was quite similar to that previously obtained under similar culture conditions with glucose as the sole carbon source by plotting q_G vs μ, 0.435 instead of 0.44 $g.g^{-1}$ *(12)*.

As shown in Fig. 2, the specific rate of malate consumption was proportional to the residual malate concentration according to the following relationship.

$$q_M = 0.31[M_r] \quad (4)$$

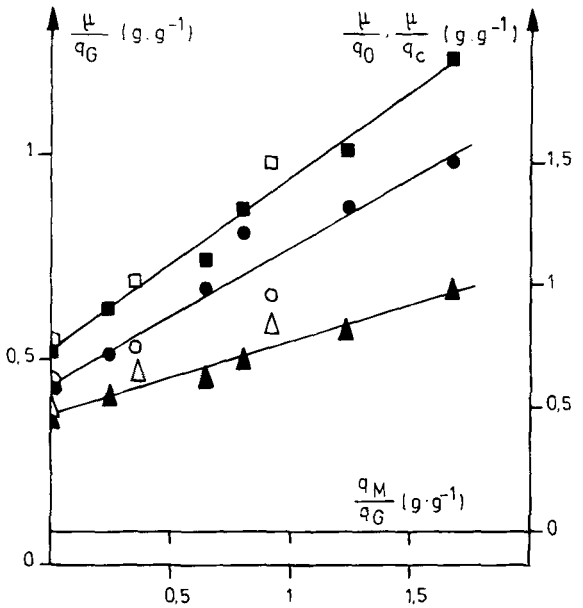

Fig. 1. Relationships between the experimental yields μ/q_j, and the q_M/q_G ratio for oxidative breakdown of substrates; G: glucose (○, ●); O: Oxygen: (△ ,▲); C: Carbon dioxide (□, ■); M: Malic acid. Open symbols: $\mu = 0.06\ h^{-1}$; closed symbols: $\mu = 0.12\ h^{-1}$.

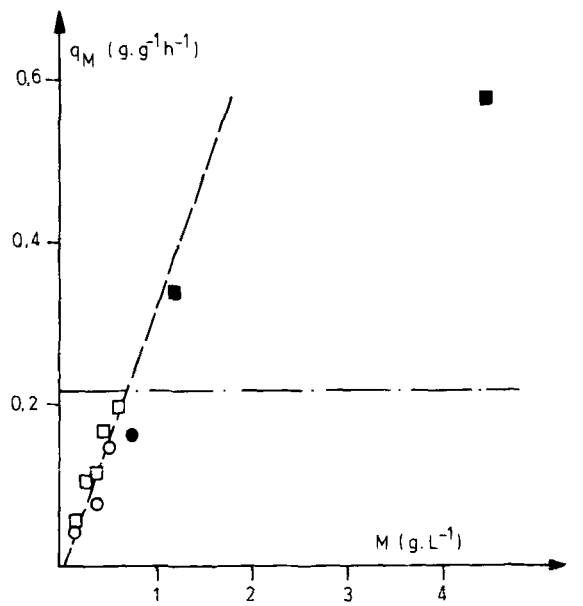

Fig. 2. Specific rate of malate consumption versus residual malate concentration for oxidative breakdown of substrates; —·—: limit for oxidative breakdown without ethanol formation; (○, ●): $\mu = 0.06\ h^{-1}$; (□, ■) $\mu = 0.12\ h^{-1}$. Open symbols: no ethanol; closed symbols: ethanol formation.

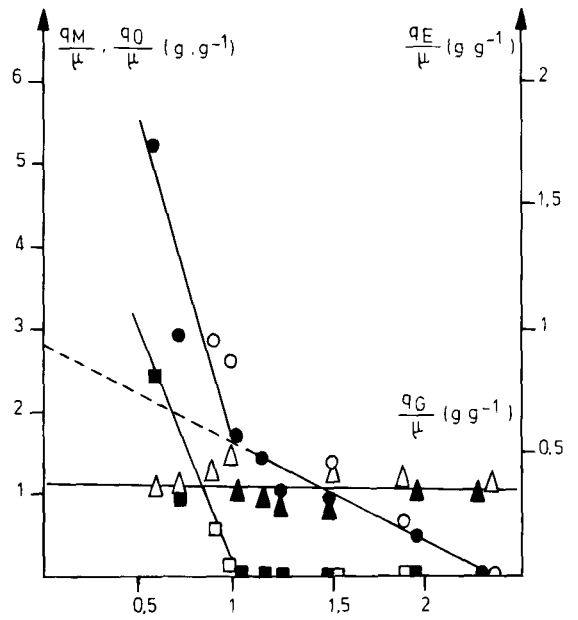

Fig. 3. Relationships between the q_j/μ values relative to substrate consumption (glucose, malic acid, oxygen) and product formation (ethanol) for oxidative breakdown of substrates; G: glucose; M: malic acid (○, ●); O: oxygen (△, ▲); E: ethanol (□, ■). Open symbols: $\mu = 0.06\ h^{-1}$; closed symbols: $\mu = 0.12\ h^{-1}$

However, this proportionality was not strictly observed for culture conditions under which a slight ethanol production occurred: deviations were more significant when the specific rate of ethanol formation was higher (*see* also Table 1). The maximum specific rate of malate breakdown achieved under strictly oxidative metabolism without an associated ethanol formation, 0.22 $g.g.^{-1}\ h^{-1}$, was higher than the V_{max} values obtained by Osothsilp and Subden *(8)* and Sousa et al. *(9)* during in vitro experiments for the Michaelis-Menten type transport of malic acid. As *S. pombe* can only metabolizes malic acid when glucose is present *(17)*, it was concluded that the rate of malate utilization was not transport limited, but was dependent on a kinetic variable of the culture, probably the specific rate of glucose consumption.

The catabolic behavior of the substrates, i.e., the oxidative breakdown with or without ethanol formation, was examined from the relationships between the specific metabolic rates relative to substrate consumption (glucose, malic acid, oxygen) and product formation (biomass, carbon dioxide, ethanol) in the form of reciprocal of the experimental yields, in fact the metabolic specific rates normalized by the specific growth rate, q/μ. (Fig. 3). Whereas reciprocal of the yield relative to oxygen, q_o/μ, remained quite constant in spite of the ethanol formation under certain experimental conditions, the coefficient relative to malate, q_{11}/μ, and to carbon dioxide, q_o/μ, (data not shown) were linear func-

tions of the coefficient relative to glucose. A change of the slope of these straight-lines was observed at a critical point characterized by a q_G/μ value close to $1/g.g^{-1}$ and a corresponding q_M/μ of 1.63 $g.g^{-1}$. It is noteworthy that, in the presence of malic acid, the experimental yield for biomass on glucose, 1 $g.g^{-1}$, was higher than the theoretical anabolic yield previously calculated for purely oxidative breakdown of glucose *(12)*, $Y_{XG} = 0.77$ $g.g^{-1}$, clearly demonstrating the participation of malate in biomass synthesis. In addition, at the critical point, which corresponds to a limit for a strictly oxidative catabolism of the substrates (without ethanol formation), by substitution of q_G by $q_M/1.63$ in Eq. (1) the specific rates of growth and malate utilization were found to be linked by the following linear relationship.

$$\mu = 0.61 q_M \tag{5}$$

As revealed by the q_E/μ profile, increased malate supply to the culture resulted in an ethanol production beyond the critical point, i.e., for q_M/q_G ratios higher than 1.63 $g.g^{-1}$. Using the whole data, the experimental yield for ethanol was deduced from the slopes of the straight-lines: ratio between slope of the q_E/μ straight-line and difference between the slope of the q_M/μ straight-line with ethanol production minus the slope of the extrapolated q_M/μ straight-line without ethanol production. The calculated value was close to the theoretical conversion yield of malate into ethanol, 0.3 instead of 0.34 $g.g^{-1}$, indicating that the ethanol formation resulted, at least partly, from an excess of malate.

Carbon Excess Environments

By plotting the experimental yields for biomass, oxygen, carbon dioxide, and ethanol on glucose vs the q_M/q_G ratio linear relationships were observed (Fig. 4A, B) and the following equations were obtained by regression analysis

$$\mu = 0.0707 q_M + 0.106 q_G \tag{6}$$

$$q_o = 0.0275 q_M + 0.0216 q_G \tag{7}$$

$$q_C = 0.617 q_M + 0.431 q_G \tag{8}$$

$$q_E = 0.271 q_M + 0.387 q_G \tag{9}$$

Whereas minor products were present in the cultures, the corresponding experimental yields are not presented: the specific production rates were in the range of 0.01 to 0.035 $g.g^{-1}.h^{-1}$ for acetaldehyde and glycerol and from 0.01 to 0.020 $g^{-1}.h^{-1}$ for pyruvate.

As shown in Fig. 5, the specific rate of malate consumption was linked to the residual concentration by the following linear relationship.

$$q_M = 0.19[M_r] \tag{10}$$

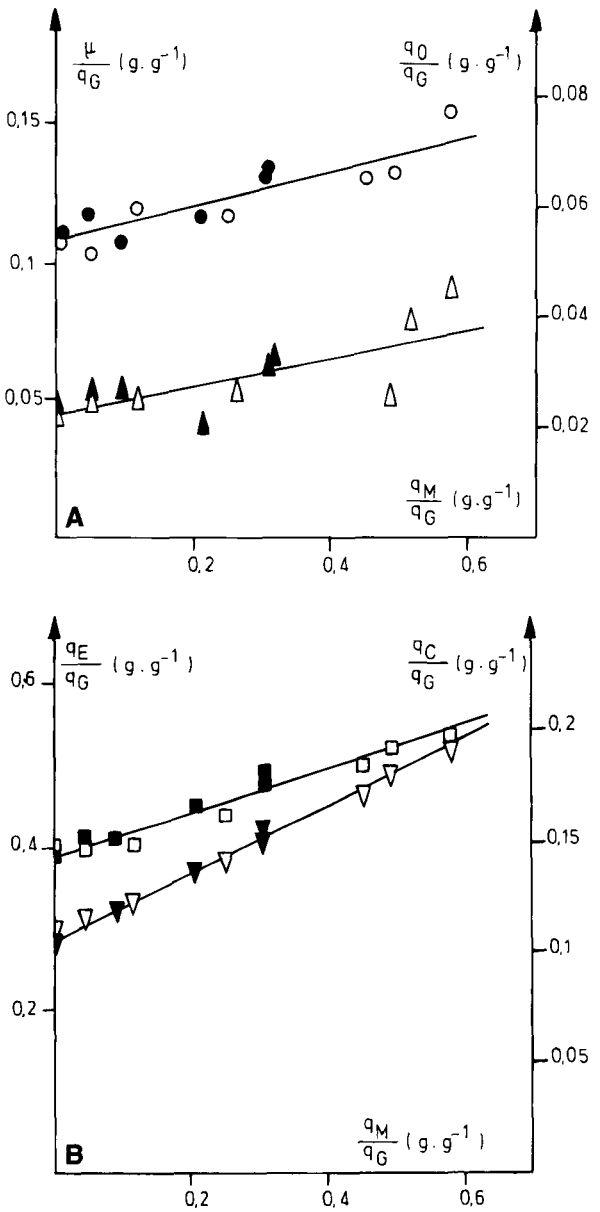

Fig. 4. Relationships between the experimental yields μ/q_j and the q_M/q_G ratio for respirofermentative breakdown of substrates; (A) substrate consumption; G: glucose (○, ●); O: oxygen (△, ▲) (B) product formation; C: carbon dioxide (▽, ▼); E: ethanol (□, ■) Open symbols: $\mu = 0.06\ h^{-1}$; closed symbols: $\mu = 0.12\ h^{-1}$.

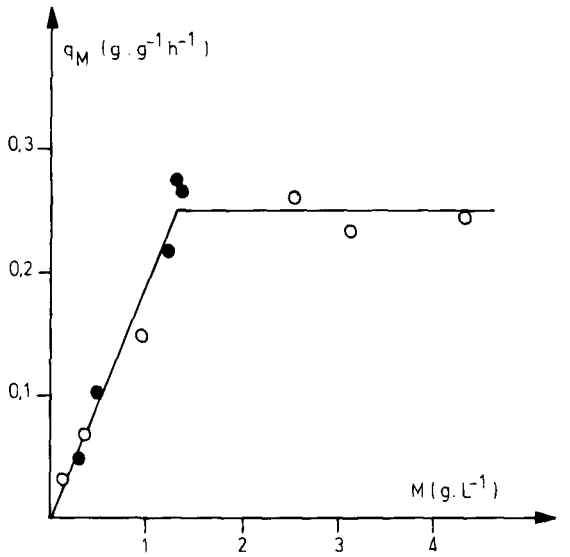

Fig. 5. Specific rate of malate consumption versus residual malate concentration for respirofermentative breakdown of substrates; (○): $\mu = 0.06\ h^{-1}$; (●): $\mu = 0.12\ h^{-1}$.

at low malate levels, but remained constant irrespective of the residual malate above 1.3 $g.L^{-1}$. In addition, it can be noted that, at a given residual malate, the q_M values were lower under carbon excess than under carbon limiting culture conditions. The change in the kinetics of malate utilization suggested that the capacity of the yeast to degrade this substrate was also dependent on another fermentation parameter, probably the specific rate of glucose consumption or, in fact, the ratio between the consumption rates of malate and glucose, as was the case under carbon limiting environments.

The relationships between the specific metabolic rates normalized by the specific growth rate are given in Fig. 6. The q_M/μ ratio increased as the q_G/μ value decreased with a concomitant significant linear decrease of reciprocal of the yield relative to ethanol formation, q_E/μ. Furthermore, a straight-line with a negative slope was obtained when q_M/q_E was plotted against q_G/q_E (data not shown) clearly indicating a progressive substitution of glucose by malate towards ethanol formation. Similar q_O/μ values were observed up to a q_G/μ of about 7.3 $g.g^{-1}$ and a corresponding q_M/μ of 2.85 $g.g^{-1}$, i.e., a q_M/q_G ratio close to 0.4 $g.g^{-1}$. As the q_O/μ value increased for higher q_M/q_G ratios, it was concluded that a part of malate in excess was oxidatively metabolized. In other words, the oxido-reductive metabolism of the cells without respiration of malate could only be maintained below a critical q_M/q_G ratio, as long as the specific rate of malate utilization remained dependent on the residual concentration. In addition, at the critical q_M/q_G value the following relationship can be derived from Eq. (6).

$$\mu = 0.134 q_G \tag{11}$$

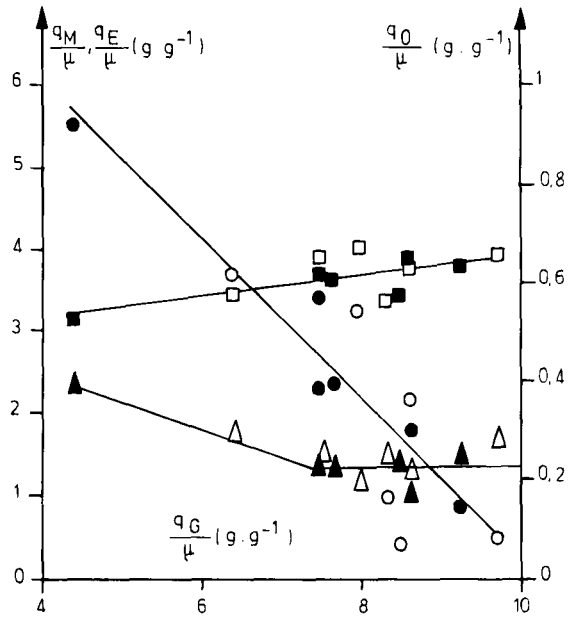

Fig. 6. Relationships between the q_j/μ values relative to substrate consumption (glucose, malic acid, oxygen) and product formation (ethanol) for respirofermentative breakdown of substrates; symbols as in Fig. 3.

In conditions under which a part of malate was respiratively metabolized, the yield for biomass on glucose was higher than the previously observed value when glucose was used as the sole carbon source, 0.134 instead of 0.117 g.g^{-1} *(17)*. As the conversion of malate into ethanol is known to proceed without energy production evidence is given for a partial participation of malate in biomass synthesis as a precursor metabolite replacing those normally derived from glucose.

CONCLUSION

Whereas the metabolic behaviour of the fermentative yeast *S. pombe* is mainly determined by glucose availability and the resulting residual concentration, in aerated cultures, the possible substitution of glucose by malic acid in central pathways was established under both conditions of carbon limitation and excess. In carbon limited environments the additional substrate is involved in cell growth, probably at both anabolic and energetic levels and the residual malate concentration is the resulting consequence of the malate supply and the needs for biomass synthesis. When supplied in excess to the cultures, malic acid is converted into ethanol and under conditions of carbon excess only a partial substitution of glucose by malate occurs. The additional substrate is mainly converted into ethanol with only weak incorporation into biomass at the anabolic level, but may also be respiratively metabolized when present in excess. However the anabolic role

of malic acid and its participation to fueling reactions during catabolism needs to be clarified. This approach, consisting of the modeling and estimation of the metabolic flux distribution in central pathways during growth of *S. pombe* on glucose or glucose-malate mixtures is now in progress.

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