cUDC 663.12:577.151.64
ISSN 1330-9862

original scientific paper

Stability of Yeast Isoenzyme Profiles in Different Growth Conditions

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Received: March 17, 1999
Accepted: October 20, 1999

Summary

Five isoenzyme systems, namely, esterase, glucose-6-phosphate dehydrogenase, acid phosphatase, alcohol dehydrogenase and lactate dehydrogenase, of yeasts belonging to five different species, Saccharomyces cerevisiae, Torulaspora delbrueckii, Pichia anomala, Schizosaccharomyces pombe and Rhodotorula mucilaginosa were analysed under different growth conditions. Two culture media were used, YEPG and modified grape must. For each medium, comparison of the isoenzyme patterns obtained in exponential and stationary phase of growth was performed. Our results showed that the growth conditions influenced differently the isoenzyme patterns of the studied strains but, under standardised conditions a good stability of patterns was achieved. Different isoenzyme patterns were obtained for the five species under study, confirming the discriminatory power of isoenzyme analysis.

Key words: isoenzyme stability, electrophoretic patterns, yeasts, growth conditions

Introduction

Electrophoretic enzyme polymorphism enables systematic and evolutionary genetic analysis and has been a current approach in molecular taxonomy of prokaryotes and eukaryotes (*1*). In yeasts, several studies evaluate the usefulness of this methodology as a taxonomic tool (*2–4*) and to type clinical isolates of pathogenic species (*5–7*). Recently, the clear distinction of the species belonging to the *Saccharomyces sensu stricto* group was achieved using the electrophoretic mobility of selected enzymes (*8*). However, a disadvantage often appointed to isoenzyme analysis is the known influence of growth conditions on enzyme activity (*9–12*). In fact, when yeast cells are grown in batch culture there is a continuous variation of population density and also substrate and product concentration. Furthermore, there are significant differences in the specific growth rates and in the metabolic process of substrate utilisation and regulation in the different yeast species, which may be reflected in the expression of enzyme activity.

In order to assess to what extent this variation may affect the stability of isoenzyme patterns, in the present study five isoenzyme systems of four ascomycetous yeast species, *Saccharomyces cerevisiae, Torulaspora delbrueckii, Pichia anomala* and *Schizosaccharomyces pombe*, and a basidiomycetous yeast, *Rhodotorula mucilaginosa* were analysed. Comparisons of isoenzyme patterns obtained in two distinct growth phases, exponential and stationary, as well as in two culture media were performed and the similarity among the different conditions was evaluated by numerical analysis.

Materials and Methods

Yeast strains and growth conditions. The yeast strains used in this study were *Pichia anomala* IGC 4121, *Rhodotorula mucilaginosa* IGC 5166^{T}, *Saccharomyces cerevisiae* IGC 4455^{T}, *Torulaspora delbrueckii* IGC 2477^{T} obtained from the Portuguese Yeast Culture Collection (PYCC),

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New University of Lisbon and *Schizosaccharomyces pombe* ISA 1190 obtained from Microbiology Laboratory, Instituto Superior de Agronomia, Portugal. Stock cultures were maintained in yeast-extract-peptone-glucose-agar (YEPG) and in grape must-agar, at 4 °C.

For yeast growth two different media were used: YEPG containing Bacto-yeast extract (0.5 % m/V); Bacto-peptone (1 % m/V); glucose (2 % m/V) and grape must adjusted to approximately 10 % (m/V) sugar content and to pH = 5.5. Growth was conducted on a rotary shaker (170 rpm), at 25 °C. To test both the influence of the media and the phase of growth on the isoenzyme patterns, cells were grown on the two media used and harvested at A_{640} previously determined, for each of the strains under study, corresponding to exponential and to stationary phase of growth. After harvesting of cells by centrifugation (5 000 × g, 4 °C for 7 min) they were washed twice with 20 mL buffer I (3.2 mM Tris-HCl pH = 7) and stored at –20 °C until further utilization.

Preparation of extracts for electrophoresis. For protein extraction, cells were disrupted with 1 g of 0.5 mm Ø glass beads and 1mL of buffer II (60 mM Tris-HCl pH = 6.8) by vortexing 1 min and cooling the tube on ice for another minute. This operation was repeated at least 12 times, until more than 70 % of the cells were disrupted. Insoluble debris and undisrupted cells were removed by centrifugation at 15 000 × g, 0 °C for 30 min. Protein extracts were stored at –20 °C.

Electrophoresis and detection of enzyme activity. Five enzymes, esterase (EST – EC 3.1.1.1), acid phosphatase (ACP – EC 3.1.3.2), glucose-6-phosphate dehydrogenase (G6PD – EC 1.1.1.49), lactate dehydrogenase (LDH – EC 1.1.1.27) and alcohol dehydrogenase (ADH – EC 1.1.1.1.), were assayed. Electrophoresis and detection of enzyme activity followed the procedures previously described by Duarte and collaborators (*8*).

For each strain, grown at different conditions, equal amounts of protein were electrophoresed, on the same gel. Experiments were repeated at least three times from different cell cultures.

Analysis of data. The relative electrophoretic mobility (R_m) of the enzyme bands was calculated as the ratio of each band migration to that of the tracking dye. These values were then corrected mathematically with the value obtained for *Sacch. cerevisiae* IGC 4072, electrophoresed on the same gel and used as a reference. Numerical analysis was performed with NTSYS-pc software package (*13*). For each enzyme, original matrices consisted of presence (1) or absence (0) of a band with a given R_m for every strain under the growth conditions tested. Relationships between electrophoretic patterns of each growth condition, including all the repetitions, were calculated using the Simple Matching similarity coefficient. Dendrograms were generated by applying unweighted pair group average linkage clustering (UPGMA) to the similarity matrix obtained. In order to test the fit of the clustering, the co-phenetic correlation coefficient was calculated (*14*).

Results and Discussion

The electrophoretic migrations obtained for the five isoenzyme systems of the strains grown on the two media and at different growth phases are shown in Fig. 1. The electrophoretic types presented are the result of several experiments and all the isoenzyme bands detected are represented including those that where not observed in all the repetitions. The similarity between the triplicates assayed for each growth condition, and among growth conditions, was evaluated by numerical analysis and dendrograms of the results obtained for each of the enzymes studied are presented and analysed individually (Fig. 2 A-E).

Esterase. Esterase patterns of *Sacch. cerevisiae, P. anomala* and *T. delbrueckii* revealed to be very stable under the different conditions tested (Fig. 2A). No variation was observed in *P. anomala* patterns and *Sacch. cerevisiae* presented few exceptions due to the presence of a very faint not reproducible band. As for *T. delbrueckii*, differences in the number of bands found and also in their intensity, in the different modalities, were detected. In cells grown on must, a very intense band was always present while in cells grown on YEPG there were only faint although reproducible bands (Fig. 1). Exponential EST of *Schiz. pombe* were different from stationary EST the last ones being poorly resolved bands with a high degree of smearing probably due to the presence of insoluble protein. As to *R. mucilaginosa*, EST isoenzymes showed to be very polymorphic. Cells collected at stationary phase of growth, in both media, presented more bands of enzyme activity than cells collected at exponential phase (Fig. 1). In the particular case of cells at the stationary phase of growth on must, patterns different from the rest, with lower enzyme activity, were found.

Regarding this enzyme, all the repetitions of the different modalities of growth of each strain were grouped together reinforcing the stability of EST under these conditions. The only exception observed was for *Schiz. pombe* where the two phases of growth appeared in different clusters (Fig. 2A).

Glucose-6-phosphate dehydrogenase. Again, *Sacch. cerevisiae, P. anomala* and *T. delbrueckii* isoenzyme patterns of G6PD showed to be very stable under the different growth conditions in opposition to what was found for *Schiz. pombe* and *R. mucilaginosa* (Fig. 2B). The same profile was found for all the conditions of growth of *Sacch. cerevisiae* and *P. anomala*, in both cases with a single band pattern. In *T. delbrueckii* a similar pattern was found for all the modalities. However, in some cases, faint bands with lower migration rates were not reproducible (Fig. 1). In what concerns *R. mucilaginosa* an equal single band pattern was found for all the modalities except for stationary phase of cells grown on must which presented three faint bands with low reproducibility. In relation to *Schiz. pombe* we could observe a decrease in band intensity from cells grown on YEPG to cells grown on must and also from cells harvested at the exponential phase of growth to the stationary ones. For

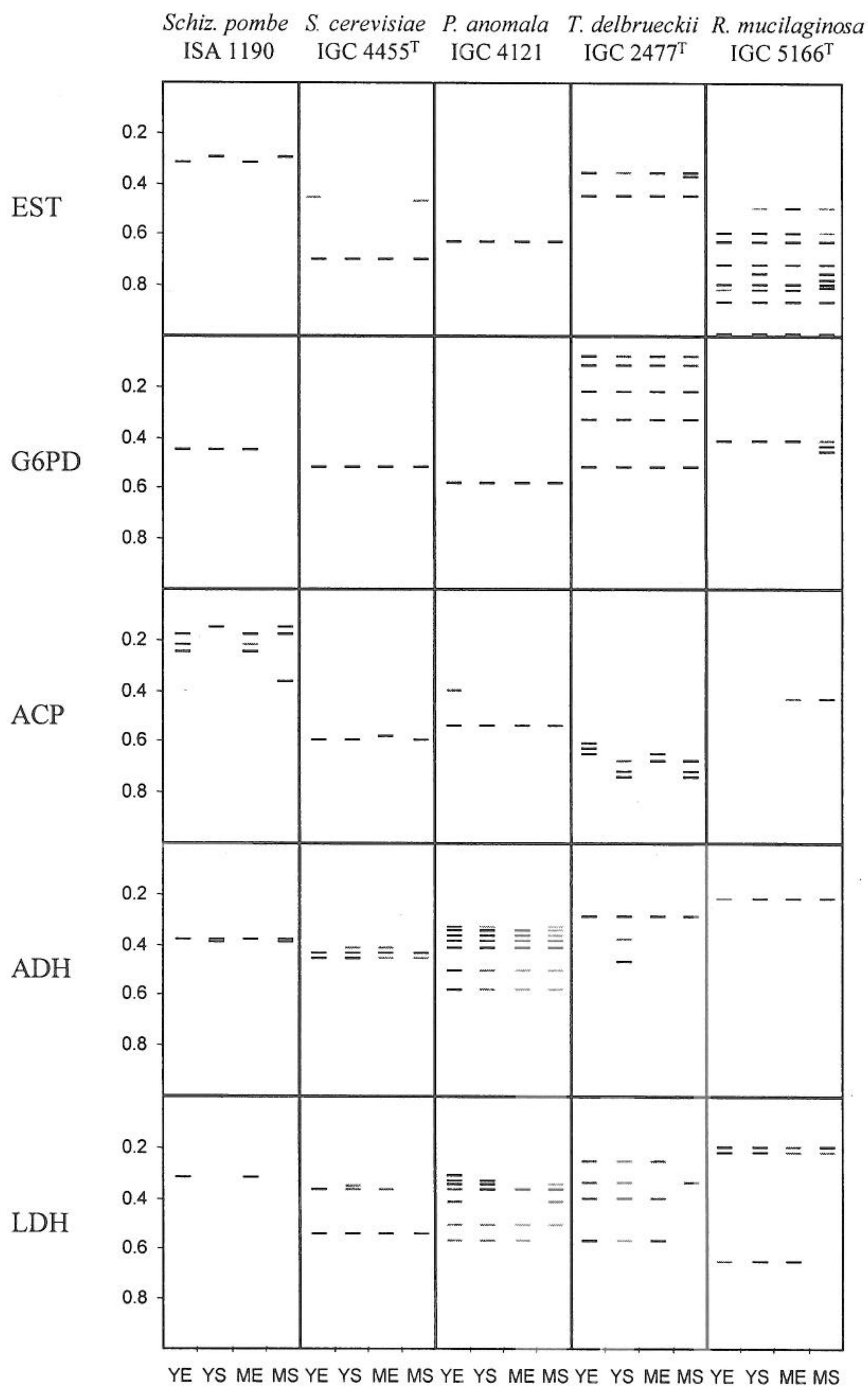

Fig. 1. Schematic representation of the electrophoretic patterns obtained for the five isoenzyme systems of the studied strains, under different growth conditions. YE-exponential cells grown on YEPG; YS-stationary cells grown on YEPG; ME-exponential cells grown on must; MS-stationary cells grown on must. Reproducible bands are represented in black; non-reproducible bands are represented in grey.

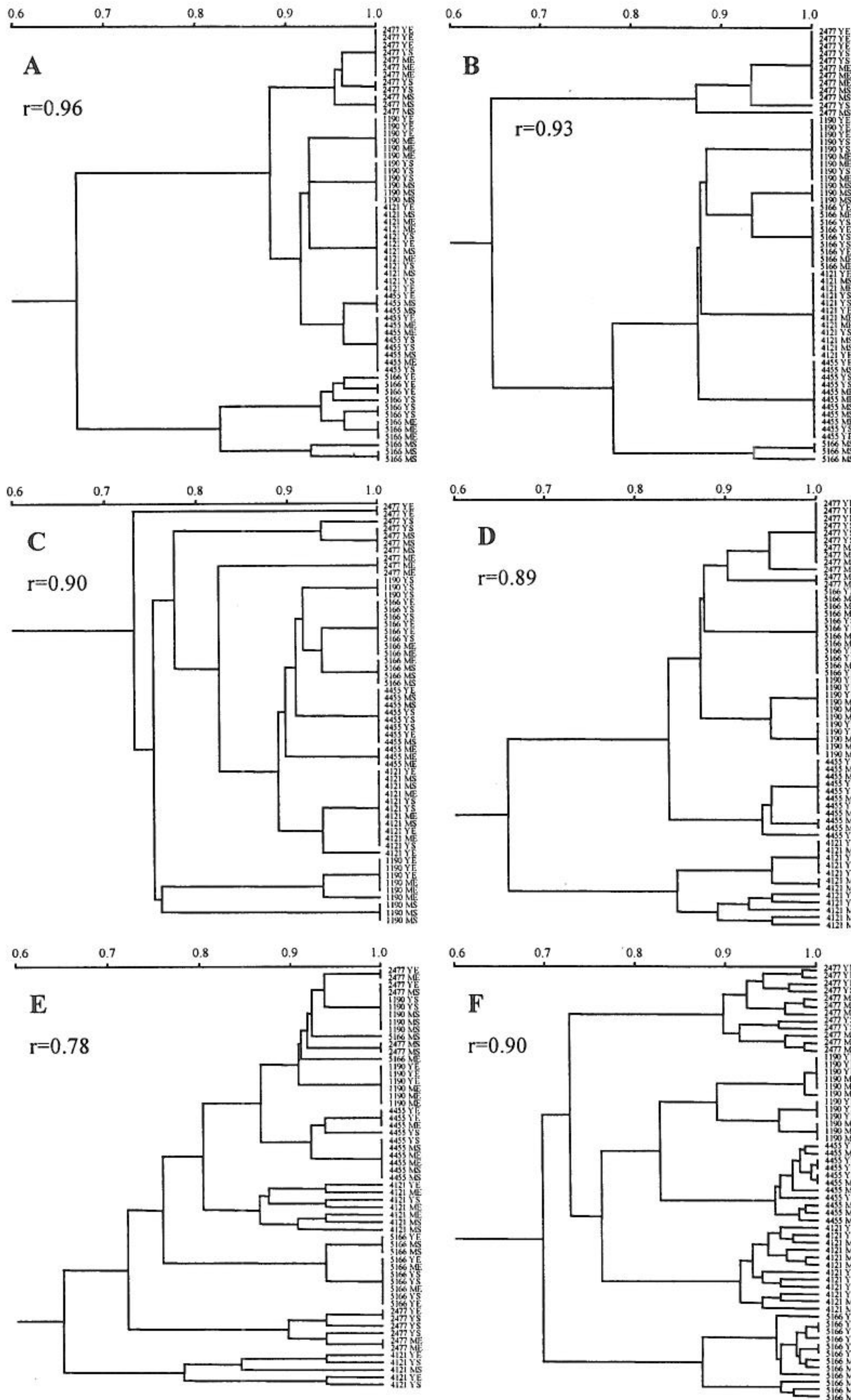

Fig. 2. Dendrogram showing the degree of similarity between the electrophoretic types of the repetitions assayed for each growth condition and among growth conditions, of the strains under study. A-EST; B-G6PD; C-ACP; D-ADH; E-LDH; F- All isoenzyme systems. YE-exponential cells grown on YEPG; YS-stationary cells grown on YEPG; ME-exponential cells grown on must; MS-stationary cells grown on must. See Materials and Methods for strain designations and abbreviations of enzymes. Scale at top represents a numerical measure of similarity. r- cophenetic correlation coefficient.

cells at stationary phase grown on must no G6PD activity was ever found. These results are not in agreement with a previous report from Tsai and co-workers (*12*) who measured activities of several enzymes in *Schiz. pombe* in exponential and stationary phases of growth and found a higher G6PD activity in cells harvested at stationary phase than in cells at exponential phase.

Acid phosphatase. Expression of acid phosphatase activity seemed to be more influenced by the growth conditions than the enzymes previously analysed (Fig. 2). In relation to *P. anomala* no difference was found among the patterns obtained for the four modalities. A slight smear appeared in only one repetition of YEPG exponential cells with one poorly defined band. A decrease in ACP activity in the presence of phosphate in the medium and a major enzyme activity for high glucose concentrations were observed by van Rijn and co-workers (*10*) for *Sacch. cerevisiae.* In the present work, we have found equal single band patterns for all the modalities except for exponential cells grown on must, which presented a band with a lower migration rate, and no qualitative differences in activity were detected (Fig. 1). No ACP activity was found for *R. mucilaginosa* cells, grown on YEPG. When growth was on must, very faint bands were detected although, for exponential cells, low reproducibility was observed. Slow migrating bands, with poor resolution, were found for all the modalities of growth of *Schiz. pombe*, which may indicate that the protein was glycosylated. Exponential cells grown on both media showed similar patterns and a different single band pattern was found for YEPG stationary cells. A different pattern but with bands in common with the other modalities was found for stationary cells grown on must. In this last case a fast migrating band was detected, which probably represents the unglycosylated or only partially glycosylated protein, observed by Schweingruber and collaborators (*11*), in a study about ACP glycosylation in *Schiz. pombe.* An increase of ACP activity during exponential growth was found by Dibenedetto (*9*), that activity remaining constant during stationary phase. This author also verified a higher ACP activity, when growth was conducted on a low-phosphate medium. In relation to *T. delbrueckii* similar patterns were found in stationary cells grown on both media. In what concerns exponential cells, those grown on YEPG presented a consistent, but completely different pattern and cells grown on must presented a pattern with bands in common with other modalities.

Alcohol dehydrogenase. From the enzymes tested in this study, ADH seemed to be the less influenced by the growth conditions as, in spite of some variation, the various patterns obtained for each strain, under the different modalities, appeared clustered together (Fig. 2D). *R. mucilaginosa* presented consistent and equal patterns of ADH for all the experiments (Fig. 1). Four ADH forms, commonly referred as I, II, III and IV, have been identified for *Sacch. cerevisiae* which are expressed under different growth conditions (*15*). In the present study, equal patterns were found in all the modalities, although some additional fainter bands appeared, with low reproducibility. An increase of ADH activity was found by Chapman and Bartley (*16*) when cells were shifted from 0.9 % glucose, aerobically, to 10 % glucose, anaerobically. In our conditions and taking into account only band intensity no difference in ADH activity was detected. Equal ADH single band pattern was found for *T. delbrueckii* in both phases of growth on must and YEPG exponential phase. In stationary cells grown on YEPG three bands were detected one of them faint and with poor reproducibility. In *Schiz. pombe* different patterns were obtained for cells at the two growth phases. Exponential cells presented much higher activity and stationary cells presented two bands of activity with very close migration rates. Our results are in agreement with the work mentioned above from Tsai and co-workers (*12*) who found higher values in exponential cells. Furthermore these authors also report the evidence for two ADH forms in stationary cells. *P. anomala* presented a higher number of bands, most of them with low reproducibility. Only two bands were common to all of the experiments (including repetitions) and four bands were present at the majority of them, what can be confirmed by the high level of similarity among the experiments shown in Fig. 2D. Higher ADH activity was found for *Sacch. cerevisiae* wine strains than for *P. anomala* and *T. delbrueckii* during fermentation in a medium designed to mimic grape must (*17*). In the present work, an increase in ADH activity from *T. delbrueckii* to *Sacch. cerevisiae* and to *P. anomala* has been noticed. Differences of ADH activity among wine yeast strains of *Sacch. cerevisiae* have also been reported and are described as being related to ethanol tolerance (*17,18*).

Lactate dehydrogenase. LDH was the enzyme presenting a higher degree of variation either between modalities and even within the different repetitions (Fig. 2E).

In *Sacch. cerevisiae* although there was a common band in all the repetitions of all the modalities of growth, additional non reproducible bands were also present except in stationary phase of growth on must. In respect of *Schiz. pombe* an identical single band pattern was present for exponential cells grown on both media tested, and no LDH activity being found in stationary cells. Similar patterns were found for all the conditions tested in *R. mucilaginosa* although some bands were not reproducible. The observed pattern had three bands, one of them, never present at stationary cells of growth on must, had a much faster migration than the other two. Both *T. delbrueckii* and *P. anomala* showed very polymorphic patterns with, in each case, many bands in common in the different modalities, most of them not reproducible.

Overall analysis of isoenzyme patterns. The combined analysis of the results obtained for all the enzymes studied in the five yeast species and in the different growth conditions enabled the construction of the dendrogram presented in Fig. 2F. Its analysis showed that all the experiments and repetitions of the different growth conditions tested, for each strain, were clustered together. When analysed globally, isoenzyme patterns of *P. anomala* IGC 4121 and *Sacch. cerevisiae* IGC 4455^T, presented variations which didn't seem to be related to the phase of growth or to the culture media. However, in the last strain, some grouping occurred for the patterns obtained in exponential cells grown on must. As for *T. delbrueckii* IGC 2477^T, in spite of the high similarity of the isoenzyme patterns observed, some influence of the

growth conditions could be seen. The cluster obtained had two principal branches, one grouping essentially exponential cells and, the other, stationary cells. Both of them were still subdivided according to the culture media. In what concerns *R. mucilaginosa* IGC 5166^T, a clear separation between the isoenzyme patterns registered for exponential cells of growth on must and the other growth conditions, was obtained. From the five species examined we could see that *Schiz. pombe* ISA 1190, isoenzyme patterns were the most influenced by the growth conditions, specially the phase of growth. Two main branches were observed, one grouping the results obtained for cells at exponential phase which were very similar for both media and another grouping the stationary cells. In this last case two minor branches could be seen, one corresponding to growth on must, clearly separated from growth on YEPG.

Conclusions

From this analysis we can conclude that the isoenzyme patterns of the strains studied were differently influenced by the growth conditions tested and that the phase of growth seemed to be more important than the culture media since the differences were mainly found between exponential and stationary phases of growth. However, the global analysis of the results showed that the differences observed were not significant since clearly separate clusters were obtained for each strain in the different growth conditions. The reproducibility of the results revealed also to be very good, equal patterns of EST, ACP, G6PD and ADH being found for most of the repetitions of the different modalities.

In similar studies conducted by Royo and collaborators (*19*) on grapevines characterisation and identification based on isoenzyme patterns, these authors refer the importance to follow a standardised methodology in order to reduce the eventual environmental effects on the isoenzyme patterns. In agreement with this proposal, we think that to avoid the variations that may occur due to the phase of growth, a well designed methodology should be followed regarding the culture conditions and time of harvest of the cells. To allow comparisons between laboratories the definition of the electrophoretic types should be based on the main bands of enzyme activity, which are consistently present and non reproducible bands shouldn't be considered.

Finally, it was possible to undoubtedly separate the strains under study, which belonged to five distinct species, analysing only five selected enzyme systems, reinforcing that isoenzyme analysis is a valuable tool for the rapid diagnostic of different yeast species.

Acknowledgements

The authors are grateful for the technical assistance of M. Filomena Alemão and Prof. Ana Amaro for helpful advice with numerical analysis.

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Stabilnost pojedinih izoenzima kvasca u različitim uvjetima rasta

Sažetak

Pod različitim uvjetima rasta ispitano je pet izoezimskih sustava (esteraza, glukoza-6-fosfat dehidrogenaza, kisela fosfataza, alkohol dehidrogenaza i laktat dehidrogenaza) koji pripadaju ovim vrstama: Saccharomyces cerevisiae, Torulaspora delbrueckii, Pichia anomala, Schizosaccharomyces pombe i Rhodotorula mucilaginosa. Za uzgoj su upotrijebljena dva medija, YEPG i modificirani mošt grožđa. U svakoj podlozi uspoređivan je odnos pojedinih izoenzima dobivenih u eksponencijalnoj i stacionarnoj fazi rasta. Rezultati pokazuju da uvjeti rasta različito utječu na strukturu izoenzima u ispitanim sojevima, dok se u standardiziranim uvjetima postiže dobra stabilnost između pojedinih izoenzima. Dobiveni su različiti odnosi među izoenzimima pet ispitivanih sojeva, što pokazuje diskriminacijsku sposobnost izoenzimske analize.