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Repression of galactose utilization by glucose in the citrate-producing yeast *Candida guilliermondii*

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SUMMARY

A strain of the yeast *Candida guilliermondii* has been shown to produce citric acid from galactose to a similar extent, and at a similar rate, as from glucose. At an initial concentration of 36 g/l of either glucose or galactose, citric acid production exceeds 13 g/l. When galactose and glucose are present in a mixture, however, galactose utilization is delayed until most of the glucose has been utilized, providing evidence for catabolite repression.

INTRODUCTION

Previous research in this laboratory has been directed towards the use of whey permeate, a by-product of the dairy industry, as a substrate for citric acid production. Experiments have been performed using the filamentous fungus, *Aspergillus niger*, but the production and yield of citric acid have been poor [4]. This is largely because, in comparison with glucose or sucrose, lactose is a poor sugar source for citrate production [5]. Attempts to use a mixture of glucose and galactose, i.e., hydrolysed lactose, as the substrate have not been successful, owing to the inability of *A. niger* to produce even moderate concentrations of citrate from galactose [5]. Furthermore, there is evidence that galactose actually inhibits citrate production from glucose [6]. For these reasons, we have investigated the use of yeasts for citrate production.

We have recently reported on the production of citrate from galactose, as well as from glucose, by a strain of the yeast *Candida guilliermondii* [7], thus raising the possibility of using hydrolysed whey permeate as a fermentation substrate. However, when growing in the presence of more than one sugar, some yeasts exhibit catabolite repression, whereby high concentrations of one sugar, e.g., glucose, can prevent simultaneous utilization of other sugars. This can be a disadvantage in an industrial process since it lengthens the fermentation time and thus lowers reactor productivity. Catabolite repression of galactose utilization in both *Saccharomyces cerevisiae* and *S. carlsbergensis* is well documented [1,3], as is that of maltose utilization in *S. carlsbergensis* [2]. Panchal et al. [8] have observed glucose repression of xylose utilization in the xylose-fermenting yeasts *C. steatolytica*, *C. shehatae*, and *Pichia stipitis*. Hence, the purpose of the present research was to investigate whether there is any catabolite repression during the aerobic process for citrate production using *C. guilliermondii*. A synthetic medium was used so that sugar concentrations could be defined precisely.

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MATERIALS AND METHODS

Organism

C. guilliermondii IMK 1, a derivative of *C. guilliermondii* NRRL Y-448, was selected for increased citric acid production following ultraviolet mutagenesis. It was maintained on slopes of Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI, USA), containing additional glucose to a final concentration of 27 g/l.

Media and cultivation

For experiments to evaluate citric acid production (CAP), the medium was Yeast Nitrogen Base (without amino acids or $(NH_4)_2SO_4$; Difco Laboratories) to which was added glucose or galactose (36 g/l), NH_4Cl (0.54 g/l), histidine (0.01 g/l), methionine (0.02 g/l), tryptophan (0.02 g/l), streptomycin (0.05 g/l) and kanamycin (0.02 g/l). The medium was prepared in 15 mM potassium

phosphate buffer (pH 6.5), and was dispensed in 250-ml conical flasks containing 50 ml of medium. Flasks were inoculated from a slope and incubated at 30 °C and 150 rpm on a rotary shaker. Samples were taken twice daily, and, simultaneously, the culture pH was adjusted, if necessary, to pH 5.2 using 10 M KOH.

For experiments in which the glucose or galactose uptake was closely monitored during the 8 h immediately following inoculation, the CAP medium was used except for the glucose or galactose (1.8 g/l) and NH_4Cl (2.68 g/l) concentrations. (The low sugar concentration was used to allow accurate measurement of its uptake during short time periods.) Incubation was as described above. The inoculum was prepared from an actively growing culture in the CAP medium except for the glucose or galactose concentration (10.8 g/l); cells were harvested by centrifugation, washed twice in 15 mM phosphate buffer (pH 6.5), and resuspended in the same buffer to inoculate the experiment.

Analyses

Biomass was determined by reading the absorbance at 600 nm, followed by reference to a standard curve for expression on a dry weight basis. After centrifugation to remove cells, the supernatant liquids were analysed for citric acid and sugars using high performance liquid chromatography as described previously [4].

Selection procedure for mutants resistant to catabolite repression

The procedure was based on one described previously [1]. Washed cells were spread-plated on the CAP medium containing agar (13 g/l), and galactose and 2-deoxyglucose at appropriate concentrations. After exposure to ultraviolet light at 253 nm for 1 min, plates were incubated in the dark. Colonies which grew at a 2-deoxyglucose concentration of 3 g/l were selected and tested in the CAP medium containing glucose and galactose at 1.8 g/l and NH_4Cl at 2.68 g/l.

RESULTS

Experiments were performed to compare the use of galactose and glucose as substrates for citric acid production, using an initial sugar concentration of 36 g/l. In addition, a mixture of glucose and galactose, both at 18 g/l, was investigated. Figs. 1 and 2 show the utilization of either glucose or galactose and the citric acid production data, respectively, plotted against the integral of biomass dry weight-time (the integral of biomass dry weight-time was determined by measuring the area beneath the curve on a plot of biomass concentration (g/l) against time (h)). In Figs. 1 and 2, the slope of the curve is a measure of the specific rate, expressed as g per g biomass per h. It is evident that the specific sugar consumption rates and citric acid production rates were approximately equal during growth on glucose and galactose. The citric acid production rate was 0.11 g/g·h, and the initial sugar consumption rate was 0.35 g/g·h. In both cases, the specific growth rate was 0.06–0.07 h^{-1}. The maximum citric acid concentrations approximated 13 g/l, after 5–6 days incubation, representing a yield of 0.37–0.38 based on sugar utilized. After the sugars had been exhausted, some consumption of citric acid was observed.

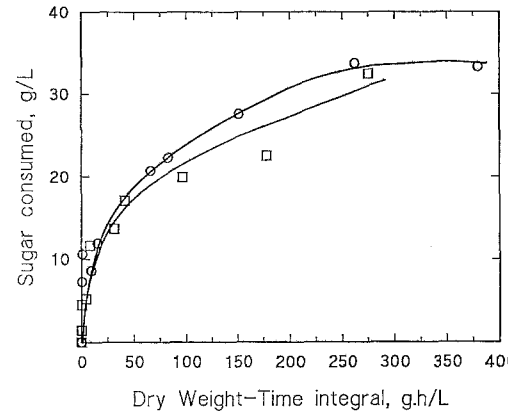

Fig. 1. Plots of glucose consumed (○), g/l, and galactose consumed (□), g/l, by *C. guilliermondii* IMK 1 when grown on glucose or galactose (initial concentration 36 g/l) against the integral of biomass dry weight-time, g·h/l. The slope of the curve represents the specific consumption rate, g/g biomass·h.

When a mixture of glucose and galactose, each at 18 g/l, was used as the substrate, marked differences were observed in their uptake.

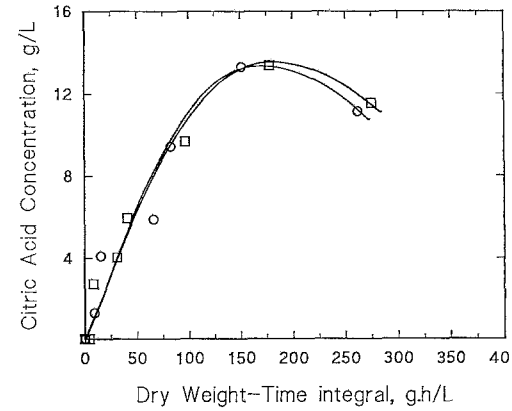

Fig. 2. Production of citric acid (g/l) by *C. guilliermondii* when grown on glucose (○) or galactose (□) (initial sugar concentration 36 g/l), plotted against the integral of biomass dry weight-time, g·h/l. The slope of the curve represents the specific citric acid production rate, g/g biomass·h.

Fig. 3 shows the sugar utilization plotted against the integral of biomass dry weight-time, as in Figs. 1 and 2. The slopes of the curves, representing the specific rates of sugar consumption, reveal that although glucose was used initially, galactose was not utilized until the residual glucose concentration was less than 4 g/l. This is evidence for catabolite repression. However, neither the specific glucose consumption rate nor the specific citric acid production rate during glucose utilization were affected by the presence of galactose (data for glucose alone at an initial concentration of 18 g/l not shown). When galactose consumption commenced, its specific consumption rate was identical to that observed when galactose was present on its own at an initial concentration of 18 g/l (data not shown). The total citric acid production and yield from the mixture were almost identical to those observed when each sugar was used alone at 36 g/l.

To monitor sugar uptake accurately during the initial growth phase, experiments were performed in which the initial sugar concentration was reduced to 1.8 g/l. Cells actively growing on either glucose or galactose were used to inoculate media containing glucose, galactose or a mixture of the two sugars. In all cases glucose utilization commenced without any lag period, even when the inoculum had been grown on galactose. However, when the inoculum had been grown on glucose, a lag period of 4–5 h was observed prior to commencement of galactose utilization. Fig. 4, showing plots of sugar consumed against the biomass dry weight-time integral, describes the results from two experiments where the inocula were grown on galactose. When this was used to inoculate a medium containing galactose alone, galactose utilization commenced immediately, as judged by the slope of the curve,

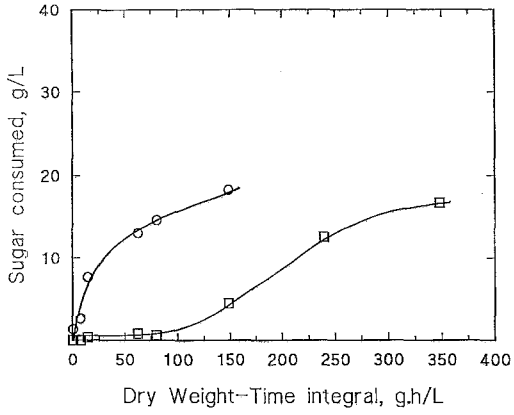

Fig. 3. Plots of glucose (○) and galactose (□) consumed by *C. guilliermondii* IMK 1 when grown on a mixture of glucose and galactose, each at an initial concentration of 18 g/l, against the integral of biomass dry weight-time. The slope of the curve represents the specific sugar consumption rate, g/g biomass·h.

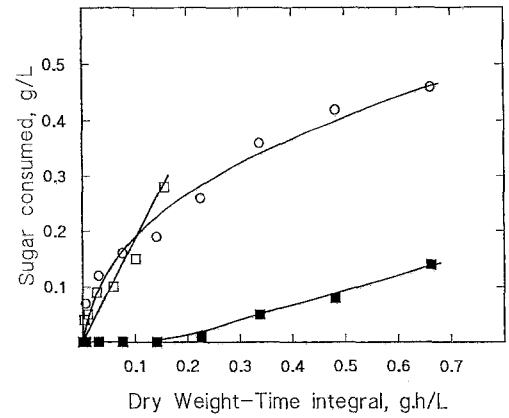

Fig. 4. Plots of sugar consumption by *C. guilliermondii* IMK 1 following inoculation with cells grown on galactose, against the integral of biomass dry weight-time. The slope of the curve represents the specific sugar consumption rate, g/g biomass·h. Galactose consumption (□), g/l; cells grown on galactose. Glucose consumption (○), g/l; cells grown on mixture of glucose and galactose. Galactose consumption (■), g/l; cells grown on mixture of glucose and galactose.

which is a measure of the specific sugar consumption rate. When this inoculum was used for a mixture of glucose and galactose, however, glucose utilization commenced immediately, but galactose utilization did not commence until after 7 h, i.e., after the glucose concentration had decreased to less than 0.4 g/l. This is further evidence for catabolite repression.

An attempt was made to isolate a mutant strain resistant to catabolite repression, using the method of Bailey et al. [1], which selects for a strain capable of producing galactose-utilizing enzymes in the presence of repressive amounts of 2-deoxyglucose. On a medium containing galactose (36 g/l) and 2-deoxyglucose (1 g/l), *C. guilliermondii* grew well. In contrast, a strain of *S. cerevisiae* failed to grow on the same medium, although it grew well in the presence of galactose without 2-deoxyglucose, thus confirming the results of Bailey et al. [1] who found a minimum inhibitory concentration of 0.1 g/l for 2-deoxyglucose. For *C. guilliermondii*, a 2-deoxyglucose concentration of 3 g/l was required before growth was prevented, suggesting that this yeast is less sensitive to catabolite repression by 2-deoxyglucose than is *S. cerevisiae*. The fact that 2-deoxyglucose at 2 g/l in the presence of galactose at 36 g/l did not prevent galactose utilization, although a clear repression effect exists at a glucose concentration down to 0.4 g/l (Fig. 4), suggests that either the catabolite repression effect is influenced by the concentration of galactose present (in effect the driving force for enzyme induction) or that 2-deoxyglucose is not effective in repressing enzymes in *C. guilliermondii*. The latter seems unlikely since

partial success has been claimed using this selection technique with other strains of *Candida* [8]. In the present work, using a 2-deoxyglucose concentration of 3 g/l, no de-repressed mutants have been isolated, despite several attempts to do so.

DISCUSSION

Investigations into the use of a mixture of glucose and galactose as a substrate for citric acid production have been stimulated by the potential use of whey permeate as a commercial raw material. The present results demonstrate that the yeast *C. guilliermondii* uses glucose and galactose equally well for citrate production, thus allowing the possibility of using hydrolysed whey permeate as the fermentation substrate (the strain of organism being used in these experiments does not utilize lactose). The data provide evidence of catabolite repression when the yeast is growing on mixtures of glucose and galactose, but the effect is not so severe that it would have a major adverse effect on the productivity.

Interestingly, the concentration of glucose at which galactose utilization is suppressed seems to vary with the galactose concentration. This suggests some competition between the two sugars for uptake/utilization, but further experiments are required to clarify this.

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