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How physiological and cultural conditions influence heterologous protein production in *Kluyveromyces lactis*

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Abstract

The optimization and scale-up of a specific protein production process have to take into account cultural conditions as well as cell physiology of growth and influence of foreign protein expression on host cell metabolism. Growth on cheap substrates, efficient secretion ability and a weaker tendency to hypermannosilate proteins than *S. cerevisiae*, make *K. lactis* an excellent and well-accepted host for heterologous protein production, even for human use. A fairly good heterologous glucoamylase yield and the setting of the optimal conditions to produce it were obtained expressing the *Arxula adeninivorans* glucoamylase in a strain of *K. lactis* and its isogenic mutant, which seems to have higher secretion ability. We performed batch cultures of both strains to analyze the influence of different physiological and environmental parameters on glucoamylase production/secretion. Interestingly, the maintenance of pH in the range of neutrality causes the consumption of a larger amount of carbon source, a longer time of production and a better stability of the active form of the enzyme, thus increasing biomass and glucoamylase production. Furthermore, the enrichment of the culture medium adds up to the action of pH control, forcing the mutant production/secretion to higher levels.
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1. Introduction

Most of the recombinant proteins produced in yeast have been expressed by *Saccharomyces cerevisiae* strains, starting from 1981 (Hitzeman et al., 1981), and it is noteworthy that the first genetically engineered vaccine for human use (hepatitis B surface antigen) was produced just in this organism (Valenzuela et al., 1982). Although most of the knowledge about genetics, physiology and cultivation techniques in yeast concerns *S. cerevisiae*, this model yeast has displayed several limitations as a host for heterologous proteins production, such as retention of the product within the cell or the periplasmic space and hyperglycosylation of secreted proteins (Buckholz and Gleeson, 1991).

Many examples testify that "non-conventional" yeasts may represent attractive systems with favorable characteristics for certain heterologous

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proteins. Among these, *Kluyveromyces lactis* has already proved to be competitive with *S. cerevisiae*: its multicopy vectors, derived from pKD1 of *K. drosophilarum* (Chen et al., 1986), show good stability in shake-flasks and pilot-scale cultures even under non-selective conditions (Buckholz and Gleeson, 1991). Moreover *K. lactis* shows a higher efficiency of secretion than *S. cerevisiae* in many cases (Gellissen and Hollenberg, 1997; Müller et al., 1998). Examples of efficient production in *K. lactis* concern both proteins of therapeutic interest, such as interleukin-1β (Blondeau et al., 1994a; Fleer et al., 1991a), granulocyte colony-stimulating factor (Hua et al., 1994), hepatitis B surface antigen (Martinez et al., 1992) and human serum albumin (Blondeau et al., 1994b; Fleer et al., 1991b; Saliola et al., 1999), and proteins with enzymatic activity, such as prochymosin (Van De Berg et al., 1990), α-galactosidase (Bergkamp et al., 1992) and glucoamylase (Bui et al., 1996b).

Glucoamylases (EC 3.2.1.3) are glycoproteins that hydrolyze starch and other oligo- and poly-saccharides removing one single glucose units from the non-reducing end of the substrate. They are used industrially for the conversion of liquefied corn starch into glucose syrup and for the production of fructose syrups and ethanol. The different glucoamylases that have been cloned from prokaryotic and eukaryotic microorganisms so far show variability in their biochemical characteristics, among which pH and thermostability are of particular relevance in biotechnological production processes (Tubb, 1986; Coutinho and Reilly, 1994). The glucoamylase from *Arxula adeninivorans* has been isolated and biochemically characterized: it shows its optimal temperature of activity at 60–70 °C and its optimal pH at 4.0–5.5 (Bui et al., 1996a).

In *K. lactis* the preference towards the utilization of glucose as carbon source is less pronounced than in *S. cerevisiae* (Breuning, 1989; Zenke et al., 1993; Mulder et al., 1995). As a matter of fact, this yeast has been used for the industrial production of the homologous enzyme lactase and for the production of low lactose milk due to the ability to grow on lactose as the only carbon source. Such large-scale processes are by now optimized. Successful heterologous protein production depends both on the genetic characteristics of the recombinant host and on the physiological conditions. The aim of the present study was to set the optimal conditions of growth and of heterologous glucoamylase production, stressing the influence of physico-chemical parameters and of the physiological status of biomass. We started from shake-flasks cultures to obtain preliminary data of production. A thoroughly investigation involved subsequently batch cultures in bioreactor.

2. Materials and methods

2.1. Yeast strains and media

K. lactis JA6 (α ade 1-600 adeT-600 trp 1-11 ura 3-12) (Breuning and Kuger, 1987) and its isogenic mutant *dgr151* were used throughout this work. The corresponding strains expressing the *AaGAA* gene contain the plasmid pTS32x-GAA (see Section 2.2) and are named JA6-GAA and *dgr151*-GAA. All strains were kindly provided by C. Donnini, University of Parma.

All experiments were conducted under selective conditions in minimum medium as described by Verduyn et al. (1992), supplemented with uracil (50 $\mu g\,ml^{-1}$), in the case of untransformed strain only, adenine (100 $\mu g\,ml^{-1}$) and triptophane (50 $\mu g\,ml^{-1}$). Different carbon sources were tested in shake-flasks experiments: glucose (20 $g\,l^{-1}$), galactose (20 $g\,l^{-1}$), lactose (20 $g\,l^{-1}$) and starch (20 $g\,l^{-1}$). All carbon sources were filter-sterilized and added to the medium just before inoculum. Lactose (20 $g\,l^{-1}$) was the only carbon source used in batch cultures. When specified medium was enriched by addition of casamino acids (1.3%, w/v, DIFCO, USA), which are acid-hydrolyzed casein maintaining selective pressure for the recombinant plasmid.

2.2. Plasmids

We have been using *K. lactis* strains transformed with the plasmid pTS32x-GAA (Bui et al., 1996b) to express the glucoamylase activity. The multicopy vector pTS32x is based on the plasmid pKD1 from *K. drosophilarum* (Chen et al., 1986) and on the bacterial vector pBluescript KS (+) (Stratagene, Germany). A SalI/SmaI DNA fragment in the plasmid pTS32x-GAA contains the *S. cerevisiae* GAP promoter (Rosenberg et al., 1990), the *A. adeninivorans GAA* gene and the *S. cerevisiae* PHO5 terminator.

2.3. Shake-flasks and batch cultivations

Yeast cultures started from frozen cells after one-night revivification in the same medium used during the experiment.

Shake-flasks experiments were conducted in 100 ml of medium in 500 ml flasks shaken at 120 rpm at 30 °C. At time 0 cells were inoculated at a biomass of 0.1 OD_{660} ml^{-1} (see Section 2.4). Experiments were performed for 110–150 h to follow the intracellular and extracellular glucoamylase activity.

Batch cultivations were performed in the bioreactor Biostat-Q-system (B-Braun, Germany) at 30 °C. A constant working volume of 0.7 l was maintained. Prehumidified air was injected at a constant flow of 1.0 l min^{-1} and a stirrer speed of 1200 rpm was kept throughout the experiments, which allowed to maintain a dissolved oxygen concentration above 20% of air saturation. Automatic addition of 2 M KOH maintained the medium at pH 6.0 during cell growth. Foaming was controlled by the addition of silicon antifoaming agent (BDH, England) to give a final concentration of 0.1 g l^{-1}.

2.4. Determination of cell density

Cell growth was monitored by measuring the optical density (OD) at 660 nm using a Lambda Bio 20 spectrophotometer (Perkin-Elmer Italia, Italy). Parallel samples varied of about 3–5%. One OD unit corresponds to a dry weight of 0.233 mg ml^{-1}.

2.5. Cell extracts

Cell extracts were prepared essentially as described by Postma et al. (1989), with the exception that cells were disrupted by agitation with glass beads on vortex (alternating 1 min in ice and 1 min on vortex for five times) instead of using sonication. Protein content of cell extracts was determined with Bio-Rad kit #500-0002 (Bio-Rad, Germany), using bovine serum albumin as a standard.

2.6. Glucoamylase assay

Glucoamylase was determined by measurement of its hydrolytic activity on starch. Starch-bound iodine has a peak of absorbance at 580 nm. Starch hydrolysis was assayed by measuring $\Delta A_{580}/\Delta t$ as described by Morlino et al. (1999). Few changes were introduced in that method: glucoamylase activity was usually estimated at 37 °C. Moreover, samples were not cooled on ice and a dilution of at least 10-fold was used in the case of enriched media to avoid interference by casamino acids. Each determination was repeated with at least three different dilutions. One unit of glucoamylase activity is defined as the quantity of enzyme needed to decrease the absorbance at 580 nm by 1 OD per minute. Enzyme assays were performed in triplicate and the values of standard deviation obtained varied of about 2–4%. No glucoamylase activity was detected in samples from the untransformed strains of *K. lactis*.

2.7. Analysis of carbon sources and extracellular metabolites

Samples were centrifugated for 2 min at 13,000 rpm to discard cellular pellet. The concentration of glucose, lactose, galactose, starch, ethanol and glycerol in supernatant was determined with R-biopharm enzymatic kits (codes: 716251, 176303, 176303, 207748, 176290, 148270; Roche, Switzerland). All samples were analyzed in triplicate and the values of standard deviation obtained varied of about 1–2%.

3. Results and discussion

3.1. Carbon source influence

Starting from the observation that *K. lactis* is able to grow on a wider range of substrates as carbon sources than *S. cerevisiae*, we first analyzed growth and glucoamylase (GAA) production on different sugars as carbon source (glucose, galactose, lactose and starch) in four different strains: JA6, its isogenic mutant *dgr151*, which seemed to gain a better secretion capacity from its mutation (Donnini et al., 2004), and the corresponding transformed strains expressing the *AaGAA* gene, JA6-GAA and *dgr151*-GAA.

No striking differences in the specific rate of growth emerged between the strains expressing GAA and their corresponding parental strains (Table 1). In the case of JA6 and JA6-GAA the highest time of duplication was detected on starch, probably due to the fact that in this

Table 1
Growth parameters of shake-flasks cultures of JA6 and *dgr151* mutant and their transformed strains (JA6-GAA and *dgr151*-GAA) on different carbon sources

	Specific rate of growth (h^{-1})				Final biomass ($mg\,ml^{-1}$)			
	2% Glucose	2% Galactose	2% Lactose	2% Starch	2% Glucose	2% Galactose	2% Lactose	2% Starch
JA6 wt	0.286	0.308	0.311	0.244	2.00	2.24	1.61	0.87
JA6-GAA	0.303	0.330	0.316	0.244	2.41	2.64	2.12	2.65
dgr151	0.249	–[a]	0.143	–[a]	2.63	–[a]	2.25	–[a]
dgr151-GAA	0.260	–[a]	0.161	–[a]	2.68	–[a]	2.57	–[a]

[a] –, not detected.

case the availability of the carbon source strictly depended on single glucose units present in the medium in the early phase of growth and subsequently on GAA secretion and activity. The mutant strain *dgr151* and, as a consequence, *dgr151*-GAA showed a substantially lower specific rate of growth in comparison to the corresponding parental strain JA6, in particular on lactose (Table 1).

We observed that the carbon source was never exhausted under all the conditions tested: the higher consumption of carbon source was detected on glucose for all the strains analyzed (68% of the initial glucose was used on the average); less than 50% of carbon source was used in all the other conditions. In fact we detected an early stopping of growth: cells entered stationary phase in 24 h and pH decreased simultaneously, reaching final values of 3.0–3.5 in 24 h, apart from the carbon source used. The final biomass reached its highest value of 2.68 $mg\,ml^{-1}$ in *dgr151*-GAA on glucose (Table 1).

The best specific production of GAA was reached on lactose rather than on glucose (Fig. 1). The GAA activity detectable in the growth medium quickly decreased to very low levels after a peak reached within the first 24 h. In fact, glucoamylase activity was decreased of almost one-half in 24 h after the peak. The experiments carried out on glucose or lactose showed the same pattern of GAA activity and always confirmed the higher production on lactose. The intracellular determination of GAA activity on stationary cells showed that there was no retention of the active protein in the cytoplasm (data not shown).

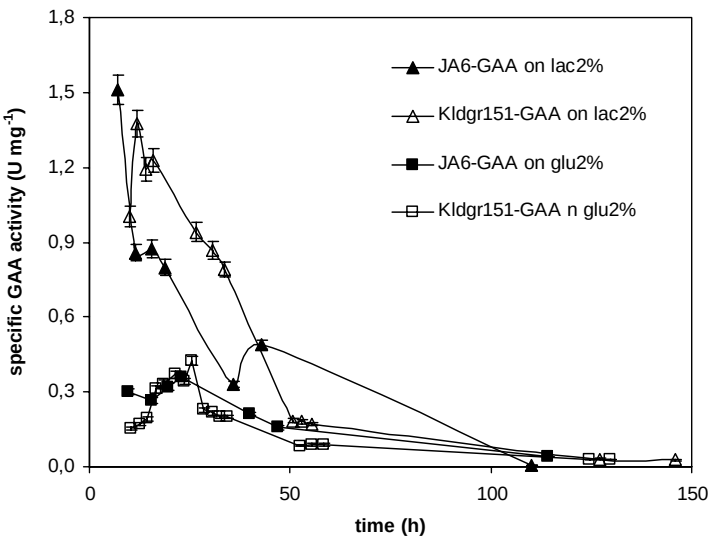

Fig. 1. Specific GAA production in JA6-GAA and *dgr151*-GAA in shake-flasks cultures on 2% glucose or 2% lactose in minimum medium.

3.2. Batch on minimum medium: pH and oxygen influence

The optimization of heterologous proteins production is not exclusively depending on the molecular strategies employed. The condition of growth as well as the effect of the expression of a foreign protein on host cell metabolism are both equally important in the development of the process. Moreover, the possibility of controlling several cultural parameters results in a much greater reproducibility and ability to reach high productivity. Batch culture is a useful tool in heterologous protein production studies to set the conditions of a specific productive process, in particular, in the viewpoint of its improvement and its subsequent scale-up. In fact, this technology allows an accurate control of cultural parameters such as oxygen concentration, pH and temperature, thus a higher reproducibility of experiment and a more accurate investigation of specific rate of growth, substrate consumption and metabolite production are characteristic of this culture method.

The incomplete utilization of carbon sources in the early experiments in shake-flasks could be caused by the exhaustion of one or more nutrients in the medium or, more likely, by some cultural parameters, which quickly change during shake-flasks cultures to unsuitable growth conditions. In particular, pH and oxygen concentration are critical parameters in shake-flasks cultures. Blondeau et al. (1994b) have already observed that pH 6.0 is optimal for biomass production in *K. lactis* CBS 683 in continuous culture at dilution rate of $0.15\,h^{-1}$. We therefore performed batch cultures of JA6-GAA and *dgr151*-GAA in minimum medium on lactose maintaining pH at the constant value of 6.0. Under these conditions both strains became able to exhaust the carbon source with a large increase in biomass in comparison to the previous experiments (Fig. 2).

JA6 is known to be a “fermentative strain”: it contains the hexose transporter gene *KHT2*, not present in other strains of *K. lactis*, and it is characterized by high glucose consumption and ethanol production rate (Weirich et al., 1997). Thus, a fine control of oxygen concentration, such as in batch cultures, may be determining in the improvement of heterologous protein production. The analysis of growth, substrate consumption and metabolite production in batch cultures both in the wild type and in the mutant strain expressing glucoamylase suggests interesting differences in the primary metabolism between JA6 and *dgr151*. Differently from JA6-GAA, no ethanol was in fact

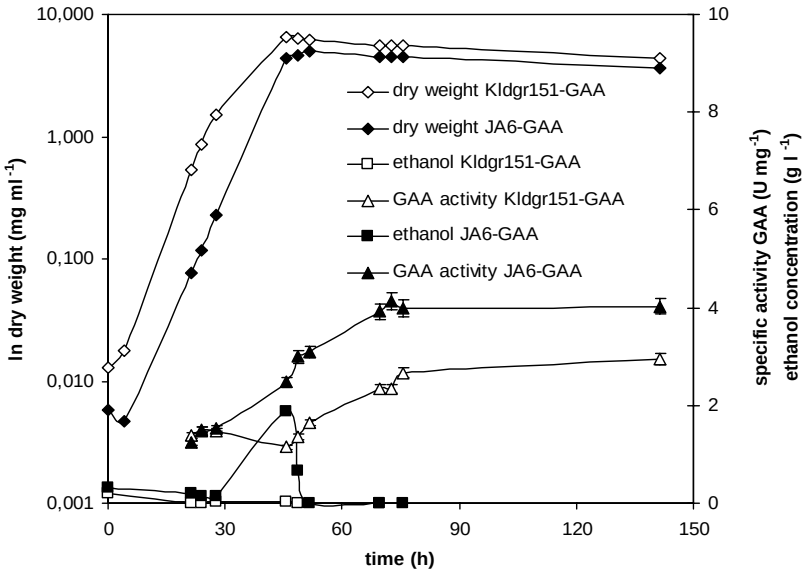

Fig. 2. Dry weight, ethanol concentration and specific GAA production in JA6-GAA and *dgr151*-GAA in batch cultures on minimum medium, 2% lactose, pH 6.0.

detected in the medium of *dgr151*-GAA cultures on lactose (Fig. 2). The yield in biomass is strictly influenced by ethanol production and as a consequence the yield in biomass was higher in the mutant strain ($0.34\,g\,g^{-1}$ in comparison to $0.24\,g\,g^{-1}$ for JA6-GAA).

Also the GAA production of both strains gains substantial advantage of pH control. The course of GAA activity in the medium of both strains showed a steep increase during the exponential phase of growth, which continued during the early stationary phase. The highest activity was therefore reached in both cases when cells had already stopped growth, unlike what happened in shake-flasks cultures. Moreover the active form of the enzyme seems to be more stable thanks to the higher pH, as we did not observe any substantial decrease in the GAA activity in the medium in 140 h (Fig. 2). As a result of the higher biomass, the longer time of production and the better stability of the active form of the enzyme, we obtained a maximum GAA production of $18.56\,U\,ml^{-1}$ for JA6-GAA and of $14.76\,U\,ml^{-1}$ for *dgr151*-GAA. JA6-GAA showed also the best production per dry weight ($4.13\,U\,mg^{-1}$ for JA6-GAA and $2.67\,U\,mg^{-1}$ for *dgr151*-GAA) and the best volumetric productivity ($3.40\,U\,ml^{-1}\,h^{-1}$ in comparison to $2.54\,U\,ml^{-1}\,h^{-1}$ of *dgr151*-GAA).

3.3. Medium enrichment

Various examples in literature suggest that the enrichment of the growth medium may improve heterologous protein production in different fungi and yeasts. For instance, examples of enhanced secretion of α-amylase after addition of asparagine in the medium are reported both for *S. cerevisiae* and *P. pastoris* (Withers et al., 1998; Kato et al., 2001; Chen et al., 2000).

We therefore performed shake-flasks cultures of JA6-GAA and *dgr151*-GAA in media containing lactose and casamino acids, which allow to enrich the medium and at the same time to maintain the selective pressure for the recombinant plasmid. Since amino acids can be used both for biosynthesis and as a source of carbon and energy, we observed an increase in the final biomass of both strains in comparison to the minimum medium (Table 2). Lactose is completely exhausted in 24 h. If we analyze the course of pH in the enriched medium, we find that the lowest value reached is 4.5 in comparison to 3.0–3.5 of minimum medium. It is likely that casamino acids are able to oppose to some extent to the decrease of pH caused by cell growth, and consequently allow the complete utilization of the carbon source and a longer exponential phase of growth.

Table 2
Final biomass and glucoamylase production values of JA6-GAA and *dgr151*-GAA in shake-flasks cultures on enriched medium, 2% lactose

	Final biomass ($mg\,ml^{-1}$)	Volumetric GAA production ($U\,ml^{-1}$)	Specific GAA production ($U\,mg^{-1}$)
JA6-GAA	6.02	21.15 ± 0.74	3.51 ± 0.12
dgr151-GAA	5.67	23.70 ± 0.55	4.18 ± 0.10

The GAA activity in supernatants showed that the highest values are reached when cells have already stopped growth both in JA6-GAA and *dgr151*-GAA cultures and we did not observe any substantial decrease in the GAA activity in the medium after more than 150 h. The best producer strain in enriched medium appears to be *dgr151*-GAA with a maximum production per dry weight of $4.18\,U\,mg^{-1}$, that is a three-fold higher specific production in comparison to shake-flasks in minimum medium (Table 2).

Batch experiments on enriched medium performed in bioreactor to control pH and dissolved oxygen completely eliminated carbon loss due to ethanol production in the case of *dgr151*-GAA with a substantial increase in biomass yield, as already observed in the corresponding cultures on minimum medium. In fact, the yield in biomass reached the value of $0.44\,g\,g^{-1}$. No substantial improvement was obtained in GAA production in batch in comparison to shake-flasks cultures, both in JA6-GAA and *dgr151*-GAA.

Comparing the data obtained in the case of *dgr151*-GAA in minimum and enriched medium in batch cultures, improvements result in the final biomass and in productivity (from 6.52 to $7.69\,mg\,ml^{-1}$ and from 2.54 to $5.64\,U\,ml^{-1}\,h^{-1}$). On the contrary, JA6-GAA glucoamylase production seems to be negatively affected by the enrichment of medium, as the maximum GAA activity per dry weight decreased substantially from 4.13 to $2.98\,U\,mg^{-1}$.

In conclusion, batch experiments pointed out the importance of maintaining the pH at 6.0 for growth and for protein production: this condition results in the complete utilization of the carbon source and, as a consequence, in a higher biomass level. This parameter proves to be important also to determine a longer stability of the active form of the *A. adeninivorans* glucoamylase. Lactose proved to be the optimal carbon source among different carbohydrates as it allows the highest heterologous protein production. The enrichment of culture medium with casamino acids results in a higher final biomass, but it improves substantially GAA secretion/production only in *dgr151*-GAA. Actually, it seems to exert some negative influence on the JA6-GAA glucoamylase production.

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