Production of Yeast Extract from Whey using Kluyveromyces marxianus

Jean P. de Palma Revillion, Adriano Brandelli and Marco A. Záchia Ayub*

ABSTRACT

The yeast Kluyveromyces marxianus B. as grow on whey to produce nucleotides in yeast extract. The second treatment resulted in yeast extracts containing large amounts of RNA. The extraction of RNA resulted in yeast extract with high levels of monosodium glutamate (MSG), inosine 5'-monophosphate (IMP) and guanosine 5'-monophosphate (GMP) which may be extracted from the biomass. These substances are efficient flavour enhancers at levels below detection thresholds (Schiffman, 1987). The chemical compounds responsible for flavour enhancement action in crude preparations were found to be predominantly due to monosodium glutamate (MSG), IMP, and GMP (Akiyama et al., 1975). These three flavour enhancers are well known in the food industry and are now commercially available worldwide. They accentuate meaty flavours in foods and have found applications in...
many savoury products. The world market of flavour enhancers surpasses US$ 1.1 billion per year (Révillion et al., 2000).

The preparation of yeast extract requires disruption of cell walls. However, among physical, chemical, and enzymatic methods for yeast breakage, autolysis is the only method that appears practical on industrial scale (Orban et al., 1994). Yeast autolysis occurs around a pH of 5.0 (Béhalová and Beran, 1979). However, during cell autolysis, intracellular RNA normally decomposes to low molecular weight non-flavourising substances such as nucleosides or puric/pyrimidic bases. Indeed, when autolysis is carried out at pH lower than 6.0, the GMP formation is very low (Tanekawa et al., 1981). However, 50-80% of intracellular RNA remained intact when yeast autolysis was developed at a constant pH range of 6.2-6.4 at 30-60°C for 10-30 h (Tanekawa et al., 1981). In this case, the remaining intracellular RNA could be easily hydrolysed to 5'-nucleotides by the action of phosphodiesterase preparations, obtained from malt roots or fungi, in a manner to obtain an extract rich in flavouring compounds such as GMP or IMP (Nagodawithana, 1992).

Microorganisms such as and dau ut s, accharomyces cerei s ae, Bac us su t s, pen c um c tr num, and M crococcus g utam cus (Belem et al., 1997; Ichii et al., 1993) have been reported to produce ribonucleotides. The yeast uyieromyces marx anus has shown great potential for cultivation on whey, due to its P-galactosidase production when lactose is used as carbon source (Ichaurrondo et al., 1993; Rech et al., 1999). Moreover, marx anus has very high growth yields when compared with other yeasts (Castillo, 1990), and it is a GRAS microorganism, being permitted to be cultivated on whey (Dziejak, 1987).

The aim of this work was to investigate the use of cheese whey as a culture medium to produce yeast extract for food use by cultivation of marx anus.

MATERIALS AND METHODS

Reagents

Alcalase™ 2.4L was from Novo Nordisk (Copenhagen, Denmark). Ribonuclease A, agarose, diethanolamine, bakers yeast RNA type III, were from Sigma Chemical Co. (St. Louis, USA). 5’-phosphodiesterase was prepared from malt roots as described elsewhere (Tanekawa et al., 1981).

Media composition

The medium used was made up with 70 g L⁻¹ of reconstituted sweet whey powder, obtained from a local dairy industry (Elege Latinios SA, Lageado, Brazil). Whey powder composition was 71% lactose, 11.1% soluble protein, 0.7% fat, 3% moisture, and 7.2% ash.

Whey hydrolysis

Enzymatic hydrolysis of whey was carried out using 0.1% (v/v) Alcalase 2.4L. The pH was corrected from 7.0 to 8.5, and the solution was incubated at 50°C for 3-4 h under agitation. Then, the pH was adjusted to 5.5, optimum for marx anus growth. Corrections of pH were done using food grade NaOH or citric acid. This avoided protein precipitation during the sterilization process.

Cultivation

Whey was inoculated with marx anus CBS 6556 to obtain an initial count of 10⁶ cells/mL. Batch cultivation was carried out on a 2.0 L stirred tank bioreactor (New Brunswick Scientific, USA). The system was incubated under agitation and sterile aeration of 7 L/min, which allowed a conversion rate of 0.55 g biomass per g lactose consumed (Moresi, 1990). Under these conditions, lactose was completely consumed within 20 h. After cultivation, biomass was recovered by centrifugation at 2,500 g for 10 min.

Cell concentration

The cell concentration was estimated by measuring the optical density at 620 nm and relating the readings to biomass dry weight with a calibration curve. The cells were harvested at 10,000 g for 5 min and washed twice with phosphate buffered solution, pH 6.6.

Mechanical disruption of cell walls

Mechanical breakage of cell envelope was carried out using a Puc-Vikosator colloidal mill (Kolloidtechnik, Germany). Cell suspensions were treated through a 0.03-0.05 mm opening for repeated cycles. Cellular envelopes were separated by centrifugation at 10,000 g for 20 min, the pellet was discarded and the supernatant was used for analysis of protein concentration.
Yeast autolysis
A 10% (w/v) cell suspension was prepared in 50 mM diethanolamine buffer pH 6.4. Ethyl acetate was added to a final concentration of 5% (v/v). Cell suspensions were then incubated at 35 or 50°C for 15 or 30 h. Yeast autolysates were centrifuged at 10,000 g for 20 min, the pellet was discarded and the supernatants were used for further analysis. Part of the supernatant was spray-dried at 135-139°C with flow rate of 2 mL/min in a bench Büchi Labortecnik spray-drier (Büchi Labortechnik, Switzerland).

Enzyme and heat treatments
Part of yeast autolysates were incubated at 95°C for 1 h, and then RNA hydrolysis was accomplished by enzymatic action of 20% (w/v) 5'-phosphodiesterase (Tanekawa et al., 1981) for 10 h at 55°C. Enzyme was inactivated by thermal treatment at 95°C for 5 min.

Analytical methods
Protein concentration was measured by the Folin phenol reagent method (Scopes, 1994). Carbohydrates were determined by the phenol sulphuric acid method (Chaplin, 1986). The RNA concentration was estimated by spectrophotometry, as described elsewhere (Delaney et al., 1975). Yeast extract composition analysis was carried out by AOAC methodology (AOAC, 1984).

Gel electrophoresis of RNA
RNA released by the different autolysis treatments was analysed by agarose gel electrophoresis. Samples were applied on 1.2% agarose gels containing 1%g ml⁻¹ ethydium bromide and run at 100V for 2h in TEB buffer (10 g L⁻¹ Tris, 1 g L⁻¹ EDTA, 5.5 g L⁻¹ boric acid). Gels were observed under UV light.

RESULTS AND DISCUSSION
Whey hydrolysis
The use of Alcalase® 2.4L, a broad range protease produced by Bacillus chelonensis, was effective in hydrolyzing whey proteins. In this process, soluble whey protein (0.8-1.1% weight) was hydrolysed to amino acids and peptides, which were readily assimilated by yeast. Therefore, whey supplementation with a nitrogen source was unnecessary. In fact, yeast extract has been already used as supplementation for nucleotide production by S. marx anus (Belem et al., 1997). Alcalase is widely used in the food industry and its hydrolysates are recognized to have a lower bitterness when compared to other proteases (Lahl and Braun, 1994).

Growth of K. marxianus on whey
Microbial growth and lactose consumption were followed during fermentation. A typical growth curve is shown in Fig. 1. The yeast S. marx anus grew well in whey reaching the stationary growth phase after 15 h. At this time, carbohydrate consumption reached minimum values. This indicated that whey disposal problems would be effectively reduced by K. marx anus fermentation. The maximum specific growth rate, $\mu_{\text{max}}$, the doubling time, $t_d$, the yield of biomass formation, $Y_{\text{XS}}$, calculated at the final growth phase were 0.62 h⁻¹, 1.12 h, 0.30 g cell/g lactose, respectively. The growth parameters were similar to those previously reported for S. marx anus grown on whey (Rech et al., 1999).

Figure 1 - Growth of K. marx anus on whey. (•) Biomass generated and (▲) lactose consumption. General growth conditions are described in the text.

Mechanical disruption of cell envelope
The mechanical cell disruption of yeast causes the release of hydrolytic enzymes, inducing the autolytic process (Babayan and Bezrukov, 1985). The colloidal mill is a widely studied mechanical system to rupture cells, presenting the best
possibilities for industrial utilization (van Gaver and Huygebaert, 1989). We demonstrated that a thermal pre-treatment (55°C ! 24h) of yeast cells eliminates the need for homogenisation with a colloidal mill to release the intracellular components, since this did not add to the final protein content of extracts (results not shown). Use of ultrasound, glass beads, or a colloidal mill did not result in significant differences in cell envelope rupture of . cerei s ae at different temperatures (Révillon et al., 1996). Treatment of . frag s biomass in concentrations ranging from 80-110 g/L, at 51-54°C for 8 h, was the most efficient to induce cell autolysis with solubilization of 51.5 % protein (Orban et al., 1994). Those authors showed that previous cell treatment with 5% sodium chloride, or mechanical rupture of cells did not modify the autolytic process. However, Baldwin and Robinson (Baldwin and Robinson, 1994) described the cell rupture with utilization of a two-step process: enzymatic treatment of cell wall with the enzyme Zimolase 20T, followed by cell disruption with homogeniser. They also concluded that 6 cycles at 95 MPa with enzymatic pre-treatment led to almost 95% cell rupture. It is relevant to consider that in their research the authors used the yeast and da ut s ATCC 9226, which presents higher mechanical resistance to disruption than . cerei s ae and . marx anus.

Yeast autolysis
In this work, assays were carried out varying some factors that induced the autolysis process, and then soluble protein concentration was measured in the extract obtained. To evaluate the influence of such factors on the efficiency of intracellular content release, . marx anus cells were incubated at 35 or 50°C for 15 or 30h. The results are shown in the Table 1. Thermal treatment did not influence significantly the release of protein. Maximum soluble protein was observed at 35°C for 15 h. This could be explained considering that ethyl acetate (5%, v/v) was an efficient permeabilizant agent, resulting in no important differences among the treatments.

Table 1 - Release of soluble protein by different autolysis procedures*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soluble protein (g/L)</th>
<th>Yield (g/g biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.4 ± 0.4</td>
<td>0.66</td>
</tr>
<tr>
<td>35°C</td>
<td>15h</td>
<td>19.4 ± 0.4</td>
</tr>
<tr>
<td>35°C</td>
<td>30h</td>
<td>18.8 ± 1.0</td>
</tr>
<tr>
<td>50°C</td>
<td>15h</td>
<td>18.8 ± 0.5</td>
</tr>
<tr>
<td>50°C</td>
<td>30h</td>
<td>18.4 ± 0.2</td>
</tr>
</tbody>
</table>

* Results are the mean ± s.e.m. of triplicate assays.

Table 2 - Composition of . marx anus extract*

<table>
<thead>
<tr>
<th>Component</th>
<th>mg g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>520.0</td>
</tr>
<tr>
<td>Fat</td>
<td>5.4</td>
</tr>
<tr>
<td>Ash</td>
<td>72.0</td>
</tr>
<tr>
<td>Moisture</td>
<td>54.0</td>
</tr>
<tr>
<td>Carbohydrate (difference)</td>
<td>348.6</td>
</tr>
</tbody>
</table>

* Results are the average of triplicate samples.

In addition, it was relevant to observe that the thermal treatments developed in the steps of extraction and hydrolysis did not result in insolubilization of nitrogen compounds, which was important in obtaining cell extracts rich in low MW nitrogen compounds and nucleotides. The nitrogen release from yeast cells ( . cerei s ae) in a buffered solution at pH 5.0 was faster when incubated at 55 and 44°C, than at 36°C (Feuillat and Charpentier, 1982). After 4h of heating at 55°C, there was no increase in the concentration of amino acids. However, at lower temperatures, the autolysis followed up to 48 h, and the nitrogen concentration released at 36°C were higher than those observed at 55-44°C. This kinetics suggested that first there occurred a passive release of nitrogen compounds from cells to media, followed by enzymatic action, which was rapidly inhibited.
at elevated temperatures. These data are in agreement to the ones observed in our work.

Composition of K. marxianus extract
The general composition of K. marxianus extract grown on whey is given in Table 2. K. marxianus extract contained a high content of crude protein and low fat and moisture contents. The product had a good level of crude protein (ca 50 %) and a low ash content (ca. 7 %). Both these factors are important for products intended for feed or food use. The composition of yeast products derived from whey revealed that generally these products were high in crude protein (Delaney et al., 1975).

Figure 2 - Analysis of K. marxianus extracts by agarose gel electrophoresis. Samples of yeast extracts obtained by autolysis at 35°C for 15 h (lanes B,C) or 30 h (lanes D,E); or autolysis at 50°C for 15 h (lanes F,G) or 30 h (lanes H,I). Lanes B,D,F,H represent samples treated with malt root 5'-phosphodiesterase. Lane A, standard RNA; lanes J,K control samples treated and non-treated with 5'-phosphodiesterase, respectively.

Table 3 - Release of ribonucleic acid by different autolysis procedures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ribonucleic acid (mg/L)</th>
<th>Yield (mg/g biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>375 ± 40</td>
<td>15.0</td>
</tr>
<tr>
<td>35°C 15h</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>35°C 30h</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>50°C 15h</td>
<td>285 ± 30</td>
<td>11.4</td>
</tr>
<tr>
<td>50°C 30h</td>
<td>430 ± 10</td>
<td>17.2</td>
</tr>
</tbody>
</table>

* Results are the mean ± s.e.m. of triplicate assays.

In conclusion, the autolysis in buffered solution at pH 6.4 ! 50°C ! 30 h was the best condition, which resulted in the release of intracellular RNA, being adequate to produce 5'-nucleotide-rich extracts with K. marxianus grown on whey. In addition, the use of malt extract (5'-phosphodiesterase preparation) was efficient to hydrolyse the RNA released during the autolysis.
Considerations to plant scale process
A diagram for 5'-nucleotide productive process is suggested in Figure 3. Whey must be stored in refrigerated tanks to avoid denaturation of protein and other compounds suitable for . marx anus growth, and also to inhibit the formation of undesired metabolites produced by other microorganisms. It is recommended to use stainless steel tanks provided with chilling and agitation devices. We suggest that the enzymatic hydrolysis should be conducted in fermentation tanks, because the need for agitation and heating are common to both steps and the thermal process is equivalent to pasteurisation process. Self-cleaning or nozzle-bowl centrifuges can be used for classification and yeast extract concentration may be conducted in flash vacuum concentrators and spray-dried if a final dry product is desired.

<table>
<thead>
<tr>
<th>Whey storage</th>
<th>Enzymatic hydrolysis</th>
</tr>
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<tbody>
<tr>
<td>Inoculation of raw material</td>
<td>Fermentation</td>
</tr>
<tr>
<td>Centrifugation of biomass</td>
<td>Thermal induction of autolysis and/or Mechanical rupture of cells</td>
</tr>
<tr>
<td></td>
<td>5'-nucleotide production</td>
</tr>
<tr>
<td></td>
<td>Elimination of cell debris</td>
</tr>
<tr>
<td></td>
<td>Concentration of cell extract</td>
</tr>
<tr>
<td></td>
<td>Drying of cell extract</td>
</tr>
</tbody>
</table>

Figure 3 - Proposed diagram for plant productive process flow.

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Received: October 24, 2001;
Revised: March 13, 2002;
Accepted: July 02, 2002.

Brazilian Archives of Biology and Technology