Brewery Waste Reuse for Protease Production by Lactic Acid Fermentation

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Summary

This study evaluated the use of three solid brewery wastes: brewer's spent grain, hot trub and residual brewer's yeast, as alternative media for the cultivation of lactic acid bacteria to evaluate their potential for proteolytic enzyme production. Initially, a mixture experimental design was used to evaluate the effect of each residue, as well as different mixtures (with the protein content set at 4 %) in the enzyme production. At predetermined intervals, the solid and liquid fractions were separated and the extracellular proteolytic activity was determined. After selecting the best experimental conditions, a second experiment, factorial experimental design, was developed in order to evaluate the protein content in the media (1 to 7 %) and the addition of fermentable sugar (glucose, 1 to 7 %). Among the wastes, residual yeast showed the highest potential for the production of extracellular enzymes, generating a proteolytic extract with 2.6 U/mL in 3 h. However, due to the low content of the fermentable sugars in the medium, the addition of glucose also had a positive effect, increasing the proteolytic activity to 4.9 U/mL. The best experimental conditions of each experimental design were reproduced for comparison, and the enzyme content was separated by ethanol precipitation. The best medium produced a precipitated protein with proteolytic activity of 145.5 U/g.

Key words: brewery waste, waste reuse, lactic fermentation, proteolytic enzymes

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Introduction

Beer is the most consumed alcoholic beverage in the world. During the beer production, four solid wastes are generated: the brewer's spent grain (derived from the grain processing), hot trub (protein coagulation that occurs during the boiling of the wort), residual brewer's yeast (microbial fermentative activity) and the diatomaceous earth (result of the beer clarification process) (1).

The world beer market is characterised by the production on large scale, resulting in the generation of significant amounts of these wastes. It is estimated that for every 100 litres of beer produced, 14 to 20 kg of brewer's spent grain (2), 0.2–0.4 kg of hot trub (3), and 1.5–3 kg of residual brewer's yeast are generated (1). Considering only the three largest producers (China, USA and Brazil), the world production of beer reaches almost 84 billion litres per year, which results in the generation of approx. 143 million kg of spent grain, 2.5 million kg of hot trub, and 19 million kg of residual yeast.

Such wastes, called brewery solid or wet wastes, have high water content, between 80 and 90 %. They also have a high content of organic matter and are rich in carbohydrates, proteins, amino acids, minerals, vitamins and phenolic compounds. Therefore, their final disposal in the environment is difficult, suggesting their use for applications in industrial bioprocesses (4–6). In previous studies, Mathias *et al.* (1,7) have studied the characteristics and composition of these wastes and their potential applications in biotechnology.

Lactic acid bacteria are microorganisms widely used in the food industry to obtain lactic acid, cheese and milk-based or whey-based fermented drinks (8). These microorganisms have high nutrient demand, requiring media rich in nitrogen and vitamins for their full activity and development. Furthermore, their metabolisms are sensitive to changes in temperature and pH (9). Thus, lactic acid bacteria have a complex system of proteases and peptidases to supply their needs for essential amino acids. These enzymes also allow them to grow rapidly in proteinaceous foods, such as milk (10).

The proteolytic systems of lactic acid bacteria isolated from different sources have been extensively studied by many researchers for many years (11–13). These systems have also been applied in protein hydrolysis and fermentation of cereal grains and leguminous seeds (14–16). Furthermore, the use of bioacidified malt in addition to base malt is common in countries that follow the Beer Purity Law, which may not include additives in the process (3), like in Germany. For example, lactic acid can be added to adjust the pH of the mash. The malt is acidified naturally by the action of lactic acid bacteria, especially of genus *Lactobacillus*, including *L. delbrueckii* ssp. *delbrueckii*, *L. delbrueckii* ssp. *lactis* and *L. fermentum* (17).

Considering all the above-mentioned factors, this work aims to evaluate the behaviour of lactic acid bacteria in complex media formulated with the three brewery wastes (brewer's spent grain, hot trub, and residual brewer's yeast) for the potential production of proteolytic enzymes.

Materials and Methods

Materials

Three solid residues of brewing process, brewer's spent grain, hot trub and residual yeast, were donated by a commercial brewery, Noi Cervejaria Artesanal, located in Niterói, Rio de Janeiro (Brazil). The content of moisture (18), minerals (19), total carbon, total nitrogen and free amino nitrogen (20,21) in the residues was determined in a previous work (8) (Table 1). All reagents used were from Sigma-Aldrich, Rio de Janeiro, Brazil.

Table 1. Composition of the brewery wastes (8)

	Brewer's spent grain	Hot trub	Residual yeast
w(moisture)/%	82.6±0.1	86.9±0.1	86.00±0.05
w(ash)/%	3.85±0.00	2.00±0.08	5.86±0.05
w(total carbon)/%	52.3±0.9	50.5±0.2	45.6±0.6
w(crude protein)/%*	26.89	48.78	52.74
w(FAN)/(mg/g)	0.36±0.03	0.22±0.02	4.09±0.04

*total nitrogen determined by Kjeldahl method using appropriate factor; FAN=free amino nitrogen in soluble fraction, in glycine equivalents

Microorganisms

A lyophilised culture of *Lactobacillus delbrueckii* ssp. *delbrueckii* (INCQS 383/ATCC 9649, Rio de Janeiro, Brazil) was used. This strain was previously selected due to its higher proteolytic activity than of six microbial cultures, namely: *Lactobacillus delbrueckii* ssp. *delbrueckii*, *L. acidophilus*, *L. casei*, *L. paracasei* (pure cultures), *L. acidophilus*, *Bifidobacterium animalis* ssp. *lactis* and *Streptococcus thermophilus*, and *S. thermophilus* and *L. bulgaricus* (mixed cultures). Selection was made by observing the growth and halo formation on casein agar (12,22,23).

The lactic acid bacteria were activated in MRS broth (de Man-Rogosa-Sharpe; Hi-Media, Rio de Janeiro, Brazil), with a final pH=6.5 (PHS 3B; PHTEK, Curitiba, Paraná, Brazil) at 37 °C under microaerophilic conditions. The microaerophilic conditions were achieved by burning a candle to consume the excessive oxygen and to release CO_2 (24), which are important factors for its activity (25).

After the cell growth, the media were centrifuged under refrigeration (model RB7-R;, Biovera, Rio de Janeiro, Brazil) at 4 °C and 8000×*g* for 15 min to precipitate the cells (15,16). The supernatant was removed, and the cells were suspended in a cryoprotectant medium containing 20 % (by volume) glycerol (Sigma-Aldrich) and stored at −20 °C (11,12). Before each inoculation, the stock culture was activated until the exponential growth phase was achieved.

Mixture experimental design

The behaviour of lactic acid bacteria in the crude brewery wastes was evaluated by a mixture experimental design with three components (x_1 – brewer's spent grain, x_2 – hot trub, and x_3 – residual yeast), as described by Cor-

nell (*26*). The mixture experimental design comprised a matrix totalling 7 experiments. All the experiments (Table 2) had fixed protein content (4 % by mass per volume), similar to the protein content in milk (*8*). Only the source of the protein varied (the waste or the mixture) and the protein composition of each waste (Table 1) was used to determine the mass to be added to the medium. In this experimental design, the pH value was adjusted to 6.5 and there was no pH control.

Table 2. Experiment formulations for the mixture and factorial experimental design

Experiment	Mixture design*			Factorial design	
	Molar fraction			Factors	
				w/%	
	Spent grain	Hot trub	Residual yeast	Protein	Glucose
E1	1	0	0	7 (+)	7 (+)
E2	0	1	0	1 (–)	7 (+)
E3	0	0	1	7 (+)	1 (–)
E4	1/2	1/2	0	1 (–)	1 (–)
E5	1/2	0	1/2	4 (CP)	4 (CP)
E6	0	1/2	1/2	4 (CP)	4 (CP)
E7	1/3	1/3	1/3		

*w(protein fixed)=4 % (mass per volume), CP=centre point

The results were used for testing a cubic reduced model:

$$\hat{y}=\dot{b}_1x_1+\dot{b}_2x_2+\dot{b}_3x_3+\dot{b}_{12}x_1x_2+\dot{b}_{13}x_1x_3+\dot{b}_{23}x_2x_3+\dot{b}_{123}x_1x_2x_3 \quad /1/$$

where y is the measured response (proteolytic activity), x_i is the component (brewery waste) and b_i is the coefficient calculated for model building.

Factorial experimental design

The best conditions determined by the mixture design were used as a starting point for a new experimental design for modelling and optimisation of proteolytic enzyme production. The effects of two quantitative factors, protein content (1 to 7 %) and glucose addition (1 to 7 %) were evaluated. These parameters were chosen according to the literature for cultivation of lactic acid bacteria in different media (*15,16,27*) and for protease production by other microbial species (*28–30*). In order to determine the mass of each added waste, the protein composition indicated in Table 1 was considered. For this experimental design, phosphate buffer medium (using reagents from Sigma-Aldrich) was used to minimise the effects of the resulting pH reduction.

Factorial design of the experiments (Table 2) was performed with two centre point runs. The results were submitted to regression analysis by a quadratic model:

$$\hat{y}=\dot{b}_0+\dot{b}_1x_1+\dot{b}_2x_2+\dot{b}_{12}x_1x_2+\dot{b}_{11}x_1^2+\dot{b}_{22}x_2^2 \quad /2/$$

where y is the measured response (proteolytic activity), x_i is the variable (protein or glucose content) and b_i is the coefficient calculated for the model building.

Fermentation and protease production

Fermentations with industrial wastes were carried out at laboratory scale using 500-mL flasks. The formulated media were autoclaved (5.07·10⁴ Pa, 121 °C, 20 min; Prismatec, Itu, São Paulo, Brazil) and the pH value was adjusted to 6.5, ideal for the cultivation of lactobacilli. The prepared media were inoculated aseptically with 10 % (by volume) lactic culture in its exponential growth phase ($A_{480\,nm}$=0.300–0.840).

The same inoculum was used for all experiments of the same experimental design. The flasks were incubated and homogenised in rotational shaker (model CT-712R; Cientec Equipamentos para Laboratório, Belo Horizonte, Minas Gerais, Brazil) with the temperature controlled at 37 °C and 100 rpm. For each formulation, samples were taken and the solid and liquid fractions were separated by centrifugation (centrifuge model RB7-R; Biovera) at 2500×*g* and 4 °C for 15 min. The proteolytic activity was measured by the azocasein (Sigma-Aldrich) method (*31*) at 6, 12 and 18 h. The productivity was also calculated, by dividing the obtained value for proteolytic activity by the fermentation time.

Determination of productivity and enzyme recovery

The best fermentation condition for each experimental design that was set to obtain the proteolytic extract was repeated to verify the enzymatic activity after 3 h of fermentation. The first purification step of the crude enzyme extract was performed by ethanol precipitation (*32*). Ethanol 95 % (Sigma-Aldrich) was added at the ratio 2:1, stirred for 30 min at 4 °C, then the material was vacuum filtered through a 3-µm membrane and oven dried. A known mass was suspended in acetate buffer (Sigma-Aldrich) and the proteolytic activity was determined.

Statistical analysis

The results were statistically evaluated through response surfaces generated from mathematical models built for proteolytic activity, and the Tukey's test was used to compare the mean values when necessary. The software used was Microsoft Excel v. 14.0 (Microsoft Corporation, Redmond, USA).

Results and Discussion

Table 3 summarises the results of the maximum proteolytic activity that was obtained after 6 h of microbial activity in each medium using both experimental designs. The mathematical models used for the determination of the proteolytic activity values shown in the following two equations were built based on the presented results.

$$PA_{MD}=0.0x_1+0.0x_2+2x_3+0.0x_1x_2+3.0x_1x_3+3.6x_2x_3+2.7x_1x_2x_3 \quad /3/$$

where PA_{MD} is the proteolytic activity obtained in mixture design (expressed in U/mL), x_1 is brewer's spent grain, x_2 is hot trub, and x_3 is residual yeast.

$$PA_{FD}=0.440+0.568x_1+0.333x_2+0.330x_1x_2+0.00x_1^2+0.130x_2^2 \quad /4/$$

Table 3. Proteolytic activity results

Experiment	Proteolytic activity/(U/mL)	
	Mixture design	Factorial design
E1	0	1.8
E2	0	0
E3	1.98	0.5
E4	0	0
E5	0.19	0.5
E6	0.08	0.4
E7	0	

t(lactic fermentation)=6 h

where PA$_{FD}$ is proteolytic activity obtained in mixture design (expressed in U/mL), x$_1$ is protein (%) and x$_2$ is glucose (%).

Proteolytic activity obtained using mixture experimental design

Protease release occurred in the first 6 h of microbial activity in some of the experiments (Table 3). The major proteolytic activity values were obtained from the experiments containing 50 % or more of residual brewer's yeast (E3, E5 and E6 in mixture design), which indicates that this residue had the strongest effect on the lactic acid fermentation and on protease production.

The presence of hot trub appears to have an opposite effect on the proteolytic activity in the medium. It is noteworthy that the hot trub contains significant amount of hop resins, which are not solubilised during the wort boiling. These compounds have bacteriostatic properties, which may have an inhibitory effect on the activity of lactobacilli during fermentation of the medium containing this waste in excess. At this point, no carbon source besides those already available in the waste composition was added to the medium. Moreover, the content of crude protein was chosen as the basis for the formulation of each medium, which contained 4 % protein. Soluble crude protein content was chosen assuming that the proteolytic activity of lactic acid bacteria (*10*) has some effect on the insoluble fraction, hydrolysing proteins that could be used as a carbon source. This assumption was confirmed when no added sugar led to the production of proteases by the microbial culture. However, this factor resulted in lower production of lactic acid in the medium. Both the brewer's spent grain and the hot trub are depleted insoluble residues generated in the brewing process. The first one is formed after the exhaustion of the soluble fractions in the preparation of sweet wort, and is composed mainly of structural insolubles and of high molecular mass proteins (*4*). The hot trub is formed due to the insolubility of coagulated proteins during boiling. Nonetheless, the residual brewer's yeast has significant chances of suffering lysis at the end of its active phase, releasing soluble compounds in the medium (*7*).

The model obtained for the calculation of proteolytic activity (Eq. 3) allowed the construction of the response surface shown in Fig. 1. The maximum activity was ob-
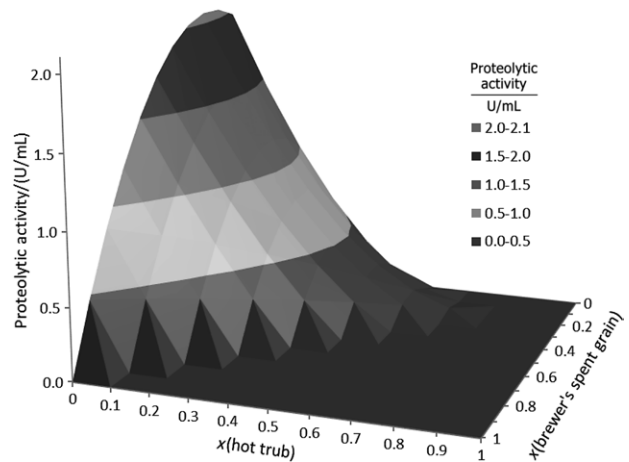

Fig. 1. Surface response for proteolytic activity of crude extract with different molar fractions of hot trub and brewer's spent grain

tained in the medium containing residual brewer's yeast as the single source of nitrogen, with a reduction of the content of any other waste. Additionally, in order to have some proteolytic activity, the medium should contain hot trub molar fraction close to zero.

The experiment E3, which contains residual yeast, led to increased proteolytic activity (1.98 U/mL) after 6 h of process, generating a productivity of 0.33 U/(mL·h), as seen in Table 3.

Proteolytic activity using factorial experimental design

The coefficients calculated for the model of factorial design (Eq. 4) allowed the evaluation of the influence of each independent factor on the expected answers. The higher the modulus of each coefficient, the greater its participation in the response calculation. The coefficient signs, positive (+) or negative (–), indicate increase or decrease in expected values, respectively. The highest coefficient and positive signal were calculated for the x$_1$ factor (protein content), indicating a significant share of the measured response (proteolytic activity). The interaction between factors x$_1$ (protein content) and x$_2$ (glucose content) is considered as significant as the effect of the factor x$_2$. The major influence of factor x$_1$ (protein content) can be observed, due to the higher calculated coefficient. The model obtained for the proteolytic activity (Eq. 4) allowed the construction of response surface shown in Fig. 2. There is an increased proteolytic activity with the increase of the two evaluated factors (protein and glucose content) since the darker colour region (indicative of the maximum enzyme activity) is located closer to the upper right side. The region of maximum proteolytic activity is located for values above 6 % protein and 6 % glucose.

Productivity and enzyme recovery results

The experiment E1 in factorial design had higher values, almost double the proteolytic activity after 3 and 6 h (4.9 and 4.6 U/mL, respectively) than the experiment E3 in mixture design (2.6 and 2.8 U/mL). Consequently, the enzymatic activity productivities were higher (1.63 and
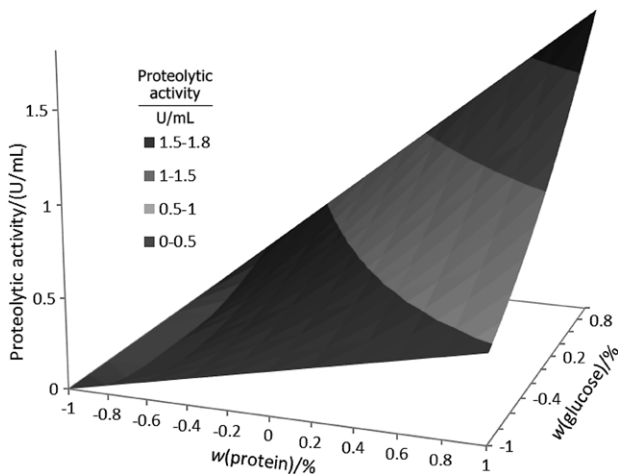
Fig. 2. Surface response for proteolytic activity of crude extract in the factorial experimental design

0.78 U/(mL·h) in the experiment E1 in factorial design than in experiment E3 in mixture design (0.85 and 0.46 U/(mL·h)). According to the Tukey's test for mean comparison, there was no significant difference at the 5 % level of significance in both experiments between the proteolytic activity after 3 and 6 h, which resulted in increased productivity over 100 % in the shorter time, 3 h. In the experiment E1 in factorial design, the protein mass was separated by precipitation with ethanol followed by centrifugation and filtration (*32*). The precipitate was suspended in 50 mM acetate buffer, pH=5.0, and the determined proteolytic activity was 145.5 U/g of precipitated protein.

The results of proteolytic activity obtained in this work were very interesting when compared with other studies in the literature of the production of proteases from different media and microorganisms (Table 4; *28,33–36*). Some authors evaluated the extract obtained from the cultivation of lactic acid bacteria in rich synthetic media, obtaining similar or lower values (*37*). However, this work presents interesting factors, such as the exclusive use of waste as nitrogen and carbon sources, the use of lactic acid bacteria, which is generally recognised as safe (GRAS), and the short fermentation time.

The addition of glucose as a source of fermentable sugar goes against the waste recovery idea, and so it was used only for the initial verification of the feasibility of using residual brewer's yeast as the sole source of nitrogen for

the formulation of the fermentation medium. There is a great potential after pretreating brewer's spent grain, a lignocellulosic material, to generate fermentable sugars, as proposed in the literature (*38,39*). Another potential use of beer residues is their use as a support for immobilisation of microorganisms to conduct fermentation (*40,41*).

The obtained proteolytic extract was submitted to a preliminary separation by ethanolic precipitation. However, downstream processes must be better evaluated for improving the efficiency of recovery and purification of proteases. Several methods of enzyme purification include separation by precipitation with sulfate or ethanol, or chromatography and dialysis (*28,34,35*).

The residual brewer's yeast, apparently the most promising alternative nitrogen source among the tested wastes, is generated as a result of microbial growth during fermentation of brewer's wort (*3*). This biomass can be reused for a limited number of times, a common practice in breweries (*42*). However, when it can no longer be reused, this biomass must be removed from the process. The amount of generated residual microbial biomass depends on the fermentation parameters, yeast species, and wort composition (*2,4*). Due to the high moisture content and rich nutritional composition, the storage of the waste requires care, generally involving a drying process and the addition of preservatives.

Final pH value

The pH value at the end of 24 h of lactic acid fermentation in each experiment of the respective experimental design is shown in Table 5.

Table 5. Final pH value after 24 h of fermentation

Experiment	Final pH	
	Mixture design	Factorial design
E1	5.2	6.4
E2	5.5	6.2
E3	5.4	6.3
E4	5.7	6.1
E5	5.7	6.3
E6	4.9	6.3
E7	5.0	

Table 4. Summary of literature data for protease production

Material	Microorganism	PA/(U/mL)	t_f/h	QPA/(U/(mL·h))	Reference
Soybean meal	*B. lichenformis*	177	72	2.4	(*33*)
Waste and synthetic medium*	*B. cereus*	180**	72	2.5	(*28*)
Synthetic medium	*B. subtilis*	236**	72	3.3	(*34*)
Wheat, soybean, cottonseed, glucose and nitrogen sources	*A. oryzae*	41** (U/g)	48	0.85	(*35*)
Wheat, soybean, cottonseed, glucose and nitrogen sources	*A. oryzae*	58.87 (U/g)	48	1.23	(*36*)

PA=maximum proteolytic activity, t_f=fermentation time, QPA=proteolytic activity productivity. *Carbon source: rice bran, wheat bran, rice husk, maize bran or crushed barley; nitrogen source: sesame cake, mustard cake, soybean cake, chickpea bran, cotton cake, urea).
**Enzymes recovered and purified by precipitation, dialysis and HPLC

There was a reduction in the pH value, mainly in the experiments in which the medium was not buffered. Even then, the proteolytic activity was probably not affected; thus, there was no reduction of pH below 5.0 (pH value of the buffer solution used in the proteolytic activity determination). The pH reduction results in a decrease in the activity of lactic acid bacteria, so the addition of a neutralising agent, such as calcium carbonate, is common in the industrial production of lactic acid (*43,44*).

Conclusions

Brewery wastes are presented as potential media for the cultivation of lactic acid bacteria, which promoted the medium acidification due to their fermentative metabolism that releases lactic acid. The presence of proteins in the medium and the absence of extra carbon source led the microorganisms to release extracellular proteolytic enzymes. The experiment containing only residual yeast showed the highest potential for producing proteases, generating an extract, purified by ethanol precipitation, with proteolytic activity of 145.5 U/g of precipitated protein.

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