

# The Role of Small Wort Peptides in Brewing Fermentations

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## ABSTRACT

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The utilisation of small peptides by brewing yeast is poorly understood despite a wealth of information on peptide transport by other microorganisms. A novel method for detection, isolation and measurement of small peptides during brewery wort fermentations was used to monitor utilisation by ale and lager yeast strains. Oligopeptide levels in wort were found to fluctuate throughout the fermentations. Measurement of extracellular protease activity provided evidence that yeast are able to continually regulate protease production in order to break down wort polypeptides into utilisable nitrogenous materials.

**Key words:** free amino nitrogen, wort oligopeptides, yeast proteases.

## INTRODUCTION

Free Amino Nitrogen (FAN) has long been regarded as a general index for prediction of healthy yeast growth, viability, vitality and fermentation efficiency, and consequently beer quality and stability. Adequate levels of the individual wort amino acids, ammonium ions and small peptides, which constitute FAN, have been shown to ensure efficient yeast cell growth and hence desirable wort fermentation performance<sup>20</sup>. A large number of studies<sup>7,8,9,15,19,20,21</sup> have examined the absorption and utilisation of wort amino acids, and we now have a clear idea of their role during fermentation. However, approximately 30% of incorporated nitrogen compounds come from sources other than amino acids. Although the utilisation of small peptides by brewing yeasts was confirmed prior to the 1950s, an understanding of their role in yeast nitrogen requirements is still limited. In this paper, we explore the role of small wort peptides in brewing fermentations. A preliminary report of this study has already been published<sup>9</sup>.

Four hundred dipeptides and up to 8,000 possible tripeptides may be found in wort, according to the number of amino acids in wort and their binding combinations to form oligopeptides with two or three residues<sup>10</sup>. Small

peptides can be used as nutritional sources of amino acids, as carbon or nitrogen sources and precursors of cell wall peptides during yeast growth, although growth is much slower when they are the sole nitrogen source<sup>5</sup>. Polypeptides are also used as a substrate because yeasts can generate proteolytic enzymes extracellularly to provide additional assimilable nitrogen to the cells<sup>11</sup>. Approximately 40% of the wort oligopeptide fraction is removed by yeast during fermentation and the peptides in beer differ from those found in wort<sup>1</sup>. However, very little is known about the range of peptides found, and generated, in wort and the order in which they are removed.

Most brewing yeast strains transport peptides with no more than 3 amino acid residues but this limit is strain dependent<sup>12</sup>. Yokota et al.<sup>24</sup> and Clapperton<sup>2</sup> reported that the concentration of low molecular weight peptides was seen to decrease during brewing fermentations. By studying fermentations that were carried out with a malt extract (a wort type prepared from malted cereals in a concentrated form), a very low attenuation rate was observed compared to that of normal gravity wort, even when the initial FAN levels were similar (200 mg/L). This suggested that regardless of the high initial FAN content of the malt extract, peptides larger than tripeptides are not utilized by yeasts<sup>14</sup>. The inability of a larger peptide to enter the yeast cells, suggests a size limit for peptide transport. It has also been demonstrated<sup>5</sup> that the L-stereoisomer's, but not the D-forms, of amino acids in di- and tripeptides are preferred substrates and also the basic amino acid-containing peptides are transported more rapidly than peptides containing acidic amino acid residues.

Studies by Ingledew and Patterson<sup>5</sup> suggested that the phase of growth and the concentration of non-peptide nitrogen might affect peptide utilization. Dipeptide transport in brewing yeasts is affected by the presence of micromolar concentrations of amino acids in the growth medium<sup>6</sup>. The presence of amino acids in the growth medium increases the sensitivity of yeast to small peptides, but not all amino acids produced the same response to the uptake of small peptides. Leucine and tryptophan appeared to be the most effective peptide uptake regulators regardless of their concentration. On the other hand, asparagine appears to be a potential inhibitor of peptide utilization. For that reason, three categories of amino acids have been identified for the effect that they induce on the uptake of small peptides. These are: amino acids that promote only slight sensitivity; amino acids that are good nitrogen sources (e.g., arginine) and repress the yeast sensitivity to small peptides, and other amino acids that are considered as inducers or accelerators of yeast peptide

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uptake sensitivity. Examination of the intracellular pools of cells grown in a medium containing amino acid inducers and small peptides showed increased levels of the peptide amino acid residues. This observation lead to speculation that these accumulated residues might bring transinhibition of amino acid uptake.

In brewing yeast cultures, the absence of competition between the individual amino acids and uptake of simple peptides, suggests that the peptide transport system is distinct from the system for amino acids. It has also been shown that di- and tripeptides share the same transport system. Patterson and Ingledew<sup>17</sup>, using a synthetic medium containing amino acids and small peptides, found that no extracellular proteinase activity was produced, which suggests that small peptides are taken up by the yeast intact, via a specific peptide transport system. The induction of simple peptide transport is mediated by a specific metabolic sensor that triggers the rapid synthesis of an additional permease or catalytic activity that is capable of modifying the existing peptide transporter system. In accordance with its role as a system providing nutrients in the form of peptides, it has been verified that the activity of this mechanism is modulated both by the quality of the nitrogen sources and the presence of amino acids included in the growth medium. Initial peptide uptake rates were observed to be higher in cells that were grown on poor nitrogen sources, such as proline, than in cells grown on preferred nitrogenous materials, such as glutamine. Based on this criterion, it has been proposed that the peptide transport system of yeasts falls under the regulatory control of the nitrogen catabolite repression mechanism<sup>18</sup>. Probably a similar inactivation as happens in the GAPI system, may take place for the peptide transport system, when yeast cells have been propagated in a medium with ammonium ions as the sole nitrogen source. Nitrogen catabolic repression most likely exerts a mild effect on peptide transport rate, whereas amino acids result in dramatic changes in initial oligopeptide uptake rates.

Moneton et al.<sup>14</sup> working with toxic and radiolabelled di- and tripeptides as medium supplements, suggested that the yeast affinity for exogenous peptide transport is highly dependent on the nature of the peptide. It was observed that di- and tripeptides that constitute methionine are actively transported into the cells, whereas equal numbers of residual peptides containing glycine were not preferred by the yeast. Studies by Marder et al.<sup>12</sup> corroborated these observations reporting that the growth responses for peptides of different sequence might reflect variations in affinity for the peptide transport system. In addition, the amino acid side chains of a peptide may determine its effectiveness as an inhibitor. Methionine peptides are very effective competitors for the utilization of other peptides. Increased lag phases are observed because of competition at the level of transport, when these peptides are used as wort supplements with other peptides. The competing peptide is taken up by the cell and hydrolyzed by endogenous peptidases. After the concentration of the competing peptide is depleted by this process, the peptide containing the required amino acid can then enter the cell and growth commences. These findings indicate that the composition of peptides is also an important determinant in the substrate specificity of the peptide permease system of the

brewing yeast strain and the initial uptake rates of peptides.

However, single peptides are not necessarily as good a source of nitrogen for growth as the amino acids that constituted them. Therefore, growth on a particular amino acid could not be used to predict the growth characteristics on the homologous di- or tripeptide. For example, the enhanced growth of yeast cells with arginine supplementation was not repeated with medium enrichment with an Arg-Arg dipeptide, and also poor cell division was observed. The negative growth response on that dipeptide could be caused by the non-transfer into the site of the peptidase activity inside the cell. Most of these peptidases are located either in intracellular organelles such as vacuoles or in the cytoplasm.

The inclusion of micromolar amounts of various amino acids in a medium that contains immobilized cells either facilitates peptide transport or it inhibits their utilization<sup>6</sup>. Other studies have confirmed this fermentation behaviour, where peptide utilization was affected by nitrogen supplementation of the medium with sources other than small peptides. For instance, the uptake of Ala-Ala was promoted 3.5-fold by the addition of leucine, while addition of asparagine resulted in a decline of the peptide consumption rate. The degree of growth enhancement and the length of the lag period prior to peptide utilization were found to be dependent on both the nature of the amino acid used and the oligopeptide quality. Although growth will be limited when the rate of nutrient peptide supply falls below that needed for optimal protein synthesis, it is difficult to understand why competition should lead to varied lag periods rather than varied growth rates and the simple competitive mechanism described by Marder et al.<sup>12</sup> appears to be inadequate to explain the specificity in the observed growth inhibitions. The good growth peptides always exhibit a slight lag phase (5 to 7 h), when their utilization is compared with individual amino acids.

Finally, it has been recognized that numerous nitrogenous materials from yeast are released into the wort during fermentation<sup>2</sup>. A significant number of these compounds are oligopeptides, which are formed during fermentation and whilst some of these may be assimilated by the yeast, others will remain in the final beer contributing to its initial flavour and long term stability. In addition, if the capacity of accumulated peptide residues is exceeded intracellularly then some of these might be released as deaminated derivatives<sup>23</sup>. The peptides remaining in the fermented wort are probably too large to be assimilated by the yeast, given the preference for peptides of 3 or fewer amino acids.

### Oligopeptides and other beer characteristics

Although the uptake and metabolism of small peptides only affects higher alcohol production indirectly, their effects on beer organoleptic characteristics cannot be ignored. Low molecular weight peptides are considered to act as foam negative effectors<sup>3</sup>. Beers containing simple peptides resulted in a significant decrease in foam stability<sup>22</sup>. A possible mechanism by which small peptides destabilize beer foam proposes that a limiting number of sites at the gas/liquid interface exist within foams, which are available for occupation by surface active molecules.

Small peptide molecules may occupy sites to the exclusion of foam enhancing polypeptides thereby resulting in a lower degree of foam stability than if all these sites were occupied by polypeptide molecules. However, addition of tripeptides to beers showed that they did not have any significant effect on beer foam stability<sup>3</sup>. Colloidal turbidity (haze) of the final beer also originates from the interaction of beer peptides with polyphenols<sup>4,16</sup>.

## MATERIALS AND METHODS

**Yeast strains:** Ale and lager yeast cultures used for this study were either strains of *Saccharomyces cerevisiae* or *Saccharomyces pastorianus*, respectively. The yeast strains employed for static fermentations were SCB8 (ale) and SCB3 (lager) obtained from Scottish and Newcastle plc. Shake flask fermentations used ale yeast No. 70 from the ICBD yeast collection.

**Fermentations:** For shake flask fermentations, a 12°Plato all malt wort was prepared in the ICBD 2 hL pilot brewery. The yeast strain that was used for the pitching of the fermentation was an ale yeast (No. 70). Fermentations were conducted at 20°C in 2 L Erlenmeyer shake flasks sealed with highly porous thin pads, in an orbital shaking incubator at speed of 150 rpm. Total wort volume was 1.5 L. Samples for analysis were collected every 24 h by using sterile glass pipettes.

For static fermentations, wort obtained from the same malt batch was used. Each wort sample (2 L) was oxygenated according to the yeast's oxygen requirements in terms of oxygen flow rate and time (e.g., 15 ppm DO for 3 min) using sterile filtered 100% oxygen. The fermentations took place in sterilized 2 L cylinders sealed with a rubber bung and a fermentation lock filled with 70% ethanol. ZnSO<sub>4</sub> solution (1.5 mL, 0.05 µM, 200 ppm with respect to zinc ions) and 40 µL of a silicon based antifoam agent were added. Aseptic techniques were observed at all times of sampling by using sterile glass pipettes. The temperature of the incubation room was 15°C for lager and 18°C for ale fermentations. All fermentations were carried out in triplicate.

### Oligopeptide determination

**1. Sample preparation:** Samples were centrifuged at 13,000 g for 15 min. Trichloroacetic acid (TCA; 3 mL; 40% w/v) was added to the supernatant (3 mL) and samples placed in ice for 60 min. Samples were re-centrifuged at 13,000 g for 10 min to remove the precipitate and the resulting supernatants were filtered through 0.2 µm syringe filters. The filtrates were stored at 4°C for 3 days to enhance further protein precipitation and once again the supernatants were collected. Samples were then frozen at -20°C awaiting future analysis.

**2. Vacuum filtration:** The cellulose ester ultra-filtration membranes (NBS Biologicals Ltd, Huntingdon, UK), used for the isolation of small oligopeptides, had a molecular weight exclusion of 500 Daltons. The membranes were washed with 20 mL of distilled water before being used and then were carefully placed at the bottom of the funnel. Small quantities of the samples (approximately 50 µL) were pipetted into the middle of the membrane trying to avoid samples being absorbed around the

perimeter. After 300 µL of sample had been filtered, the membrane pores were blocked and no further sample was able to permeate through. As the flask that was utilized to produce the vacuum was too large for the collection of the filtrates, an Eppendorf tube was attached just below the outlet of the funnel in order to collect the sample.

**3. Acid hydrolysis:** This step of the oligopeptide determination protocol is a modified version of the technique described by Dale et al.<sup>3</sup> Each sample (100 µL) was placed in a Pyrex glass tube with cold performic acid (200 µL). Each tube was sonicated for 3 min, then stored at 4°C overnight. Sodium metabisulphite (50 mg) and 7.5 M hydrochloric acid (800 µL) were added to each tube, which was sonicated again for 15 min, then placed into a heating block (110°C; 24 h). After the incubation period, the samples were each diluted to 5 mL with deionised water and filtered (0.45 µm). Each filtrate (4 mL) was evaporated to dryness using a rotary evaporator. Samples were then re-suspended in sodium carbonate (800 µL; 0.2 M; pH 9.7).

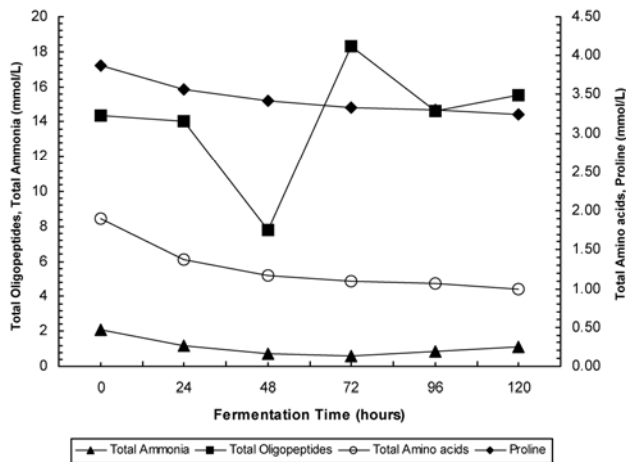
**4. Alkaline hydrolysis:** Alkaline hydrolysis was used to recover tryptophan, which was destroyed during acid treatment. Each sample (100 µL) was placed in a Pyrex glass tube. Tubes were cooled in ice and 4.2 N NaOH (5 mL) was added. Mixtures were then placed in a heating block and incubated for 24 h at 110°C. The next day, 6N HCl (5 mL) was added and the contents diluted to 12 mL with deionised water and filtered (0.45 µm). Each filtrate (10 mL) was evaporated to dryness. Finally, samples were re-suspended in sodium carbonate (2 mL; 0.2 M; pH 9.7).

**5. HPLC analysis:** HPLC analysis of hydrolysis products was carried out. Unhydrolyzed samples were also analysed to calculate the concentration of individual wort amino acids. Oligopeptide levels were estimated by subtracting the values obtained with the unhydrolyzed samples from the hydrolyzed samples.

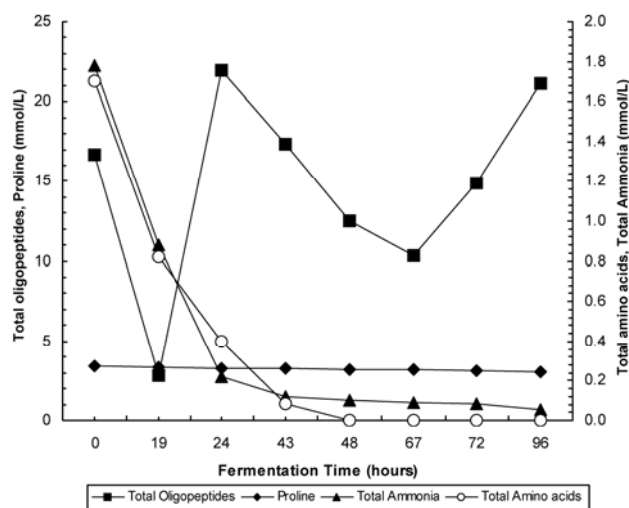
**Determination of protease activity:** General protease activity was measured employing a modified version of the method described by Mochaba et al.<sup>13</sup> For each assay, citrate-phosphate buffer (75 µL; pH 5.3) and fermentation samples (75 µL) were added to an aliquot of universal protease substrate (50 µL; resorufin labelled casein) (Roche Diagnostics Ltd, East Sussex, UK). After mixing, tubes were incubated at 37°C for 2 h. Five percent TCA (480 µL) was then added to each tube to halt proteolytic activity and the tubes incubated for a further 10 min at 37°C. Each tube was then centrifuged at 10,000 g for 10 min and the supernatant (400 µL) was added to Tris-HCl buffer (600 µL; 0.5 M; pH 8.8). The absorbance was measured at 574 nm against the blank, which instead of containing fermentation samples, contained water. The assay was calibrated with yeast proteinase A (PrA) (Sigma, St Louis, USA). The calibration curve was produced by preparing solutions of PrA from 0 units/mL to 3.75 units/mL.

## RESULTS AND DISCUSSION

A novel method was used for the detection, isolation and measurement of small peptides within wort fermentations. The fermentations employed were: 12°Plato gravity wort (1048°G) ale shake flask fermentation, and ale (SC8)



**Fig. 1.** Total nitrogen fermentation absorption profile for ale shake flask fermentations.

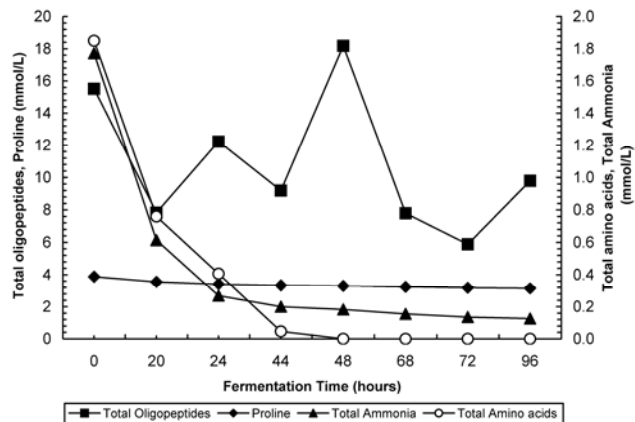


**Fig. 2.** Total nitrogen fermentation absorption profile for SC3 lager static fermentations.

and lager (SC3) static fermentations. Each fermentation was carried out in triplicate.

Figures 1, 2 and 3 illustrate the overall ale shake flask, the static lager and ale yeast fermentation nitrogen metabolic profile, respectively. The sum of the spectrum of all assimilable wort amino acids is also shown in these figures, together with ammonia and proline. This facilitates comparison of the metabolic rates of all the available wort nitrogenous yeast sources during the fermentations. During the ale yeast shaken experiments (Fig. 1), the uptake of wort oligopeptides began on the second day of fermentation, and proceeded rapidly for the next 24 h. On the third and fourth day the level of small peptides in the medium increased significantly. Thereafter, oligopeptide levels decreased once more until the last 24 h of fermentation, when the concentration started to build up again in the fermentation broth.

During the lager yeast fermentations, peptide removal from wort started very rapidly within the first 19 h of fermentation, simultaneously with amino acid consumption (Fig. 2). The wort oligopeptide concentration then started



**Fig. 3.** Total nitrogen fermentation absorption profile for SC8 ale static fermentations.

to increase until 24 h into the fermentation. Between 24 and 67 h of fermentation, oligopeptide levels fell gradually. Thereafter, small peptides started to accumulate for a second time in the fermenting wort, until the end of fermentation. The residual oligopeptide levels detected in the fermented wort were higher than the initial level in the unfermented wort (14 to 16 mmol/L).

A similar oligopeptide assimilation pattern to that of the lager fermentation was observed for the static ale fermentations (Fig. 3). In more detail, oligopeptide consumption also started during the first 20 h of fermentation and at the same time as that of amino acid and ammonium ion absorption. For the next 4 h of the fermentation, oligopeptide levels increased significantly. Between 24 and 44 h of yeast incubation, small wort peptides started to be taken up once more. During the next 4 h of the fermentation, the level of small peptides increased from 9 mmol/L to 19 mmol/L. Then, between 48 and 72 h of fermentation, the oligopeptide content declined sharply. Finally, during the last 24 h of the fermentation the concentration of single peptides increased again.

In both lager and ale fermentations, all the fermentable free amino acids in wort were consumed by the yeast within the first 48 h of fermentation as expected from previous experiments conducted by Jones and Pierce<sup>8</sup> and Lekkas et al.<sup>9</sup> However, such an effect did not take place during the shake flask ale fermentations since complete uptake of wort amino acids did not occur (Fig. 1). In all fermentations conducted, ammonia and proline utilization was incomplete as occurred in earlier work by Jones and Pierce<sup>8</sup>.

As shown in Figs. 1 to 3, oligopeptide levels in wort fluctuated throughout the fermentations. To explain this, three hypotheses were considered. The first hypothesis was that the increase in the small peptide wort concentration was induced by the low cell viability and consequently cell lysis and release of nitrogenous materials into the fermentation environment. Such an assumption was proven not to be true because cell viability even at the end of the fermentation was high (90%). In addition, the extracellular ATP levels (an index of yeast viability and cell autolysis) were found not to correlate with the pattern of oligopeptide absorption (data not shown). The second assumption that was made in order to clarify such a phe-

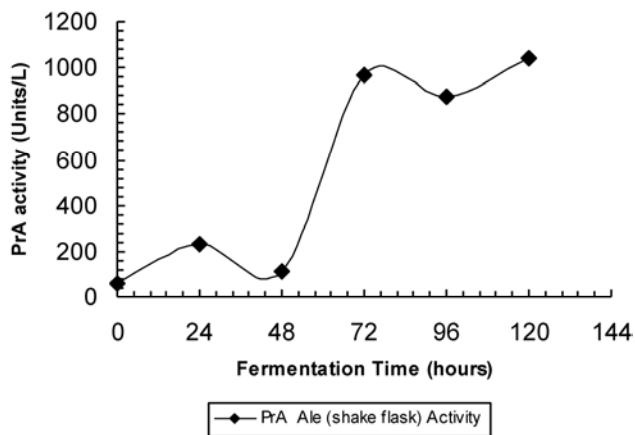


Fig. 4. Proteinase activity for the ale (shake flask) fermentations.

nomenon was that yeast cells excrete the small peptides that have already taken up, back into the fermenting wort, and then they absorb them again. However, such a theory did not seem to be applicable because when the total wort FAN content was measured, no increase in concentration was detected throughout the duration of the experiments. The last hypothesis that could provide a coherent explanation was that the excretion/secretion of yeast proteases into the fermentation environment could hydrolyse larger peptides and proteins into smaller molecules in order to supply the proliferating yeast cells with more available nitrogenous nutrients. For that reason, the overall extracellular yeast protease activity was measured.

Figures 4 and 5 show the activity of the extracellular general protease activity during the course of ale shake flask and lager and ale static fermentations. During the ale fermentations conducted in shake flasks, the activity of extracellular protease increased for the first 24 h and then decreased between 24 and 48 h of fermentation. Between 48 h and 72 h of fermentation, protease activity increased again. Thereafter, the extracellular protease activity fell until the last day of the experiment, when protease activity rose again until the completion of the fermentation.

During the static lager fermentations, protease activity increased gradually for the first 20 h then fell slightly before increasing again from 24 h until 48 h of fermentation. Between 48 and 67 h of fermentation, the protease activity remained constant and then after 72 h, it dropped sharply again before a final increase until the end of fermentation, when individual amino acid levels had been depleted. The proteinase activity during the ale fermentations displayed a similar pattern. The only noticeable difference was that the activity decreased between 48 and 72 h of fermentation, and then it started to increase again until the fermentation was complete. This was probably due to break down of larger peptides into smaller peptides, which were used by the yeast cells after all the available nitrogen sources had been exhausted. This suggests that yeast fermentative activity does not cease when free amino nitrogen is depleted, based on the observation that yeast proteases continue to be secreted/excreted, digesting polypeptides into smaller oligopeptides thus making more utilizable nitrogenous materials available.

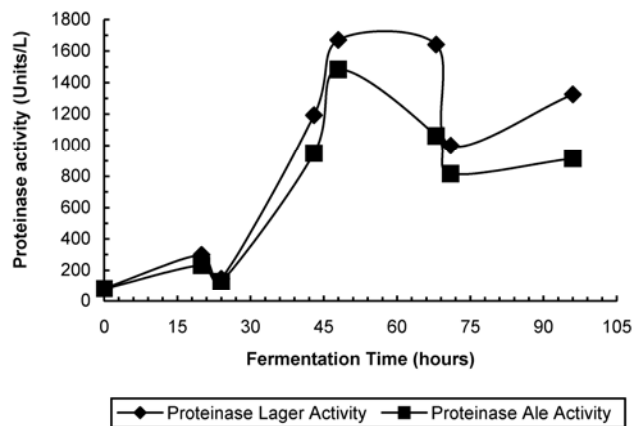


Fig. 5. Proteinase activity for the static ale and lager fermentations.

This study has shown that both lager and ale yeast strains, regardless of the experimental conditions employed in this study, could simultaneously use amino acids and small peptides as sources of assimilable nitrogen. In addition, it is believed that extracellular proteolytic enzymes are responsible for the degradation of larger wort peptides into smaller peptides in order to provide yeast cells with more available assimilable nitrogen sources.

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