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# Microbial Protease or Yeast Extract—Alternative Additions for Improvement of Fermentation Performance and Quality of Beer Brewed with a High Rice Content<sup>1</sup>

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## ABSTRACT

Reduction of production costs is an important advantage of adjunct utilization in brewing. Nevertheless, addition of 40% rice significantly decreases the biomass formation of yeast due to depletion of assimilable nitrogen in the wort. This leads to longer fermentation times and may have a negative influence on beer organoleptic quality. This study compared addition of protease to the malt-mash during mashing or supplementation of yeast extract in wort after boiling to increase the wort assimilable nitrogen content. In both cases, the fermentation was considerably accelerated, and the beer quality was significantly improved. Yeast extract gave better results than microbial protease. The fermentation kinetics and the beer quality from a 60% malt-40% rice wort supplemented by yeast extract were the same as those from a 100% malt wort.

**Key words:** Assimilable nitrogen, Brewing adjunct

## RESUMEN

**Proteasa microbiana o extracto de levadura- Suplementos alternativos para la mejora del rendimiento durante la fermentación y de la calidad de la cerveza elaborada con un alto contenido de arroz**

La sustitución parcial de la malta en el proceso de fabricación de la cerveza, presenta importantes ventajas en la reducción de los costos de producción, pero la adición de 40% de arroz reduce significativamente el crecimiento de la levadura a causa de la disminución que origina en la concentración de nitrógeno asimilable del mosto. Esto conduce a fermentaciones más lentas y puede influenciar negativamente la calidad organoléptica de la cerveza. En este estudio se compararon dos soluciones para aumentar la concentración de nitrógeno asimilable de un mosto preparado con una mezcla de malta (60%) y arroz (40%): adición de una proteasa durante la maceración y adición de extracto de levadura al mosto antes de la fermentación. En ambos casos, la fermentación fue más rápida y la calidad de la cerveza mejoró. El extracto de levadura dio mejores resultados que la proteasa microbiana. La cinética de la fermentación y la calidad de la cerveza hecha con el mosto que contenía un 60% de malta, un 40% de arroz y el extracto de levadura fueron casi las mismas que las de un mosto preparado con 100% malta.

**Palabras clave:** Nitrógeno asimilable, Adjunto cervicero

Adjunct utilization is still an important challenge in the brewing industry, particularly in tropical countries, where the malt is imported. Nowadays, 90% of the beer in the world is produced by mixed grists of malt and adjunct (25).

In South Asia, rice is the most commonly used cereal because of its large cultivation area. This cereal is traditionally considered a brewing raw material and, in some countries, it is employed at a ratio of 30–50%. This adjunct may be used to decrease production cost, to improve beer stability due to its reduced content of proteins and polyphenols, and to provide deliberate palate shifts for new products (18).

Nevertheless, high adjunct use can influence wort quality. Generally, adjuncts make little contribution to the nitrogen content of wort, serving mainly to dilute all of the noncarbohydrate components while increasing wort gravity (5). This observation has been confirmed by many studies (18,26,30,34). In fact, rice use was shown to be one of the simplest methods for lowering the assimilable nitrogen concentration of wort (30). However, quantitative results have not been reported at high levels (40%) of rice addition.

Assimilable nitrogen for brewing yeast is composed mainly of ammonium nitrogen ( $\text{NH}_4^+$ ) and especially free amino nitrogen ( $-\text{NH}_2$ ) such as in amino acids and small peptides (32). These compounds are essential for the growth and metabolism of yeast. The kinetics of fermentation, the formation of metabolic by-products, and the elaboration of beer aroma are determined, in part, by the qualitative and quantitative composition of the nitrogen fraction of the medium (11). Wort with low assimilable nitrogen ferments incompletely or becomes “stuck” due to the premature cessation of yeast growth (5). This phenomenon has been observed not only in brewing but also in wine-making (11).

To increase the concentration of assimilable nitrogen, two strategies were proposed. One was to supplement protease enzymes in malt-mashes to intensify the proteolysis during mashing (23). Nowadays, the microbial enzyme market provides a large choice of products for brewing. The second strategy was to supplement assimilable nitrogen compounds in wort at the beginning of fermentation (19).

Another solution practiced in the United States and Latin America is using a malt with a high degree of proteolysis. For example, one major product is produced with 40% rice and 60% malt grits, including a mixture of six- and two-row malting barleys. However, proteolytic malt has a lower extract and is normally more expensive than two-row barley Pilsen malt, which is commonly used in most countries. In South Asia, proteolytic malt has not been imported, and so the two previous strategies are considered the technological solution for overcoming a nitrogen limitation.

This study compared the effectiveness of these two strategies applied to wort fermentation from a malt-rice grist. First, our research focused on fermentation kinetics, particularly on yeast growth, substrate assimilation, and production of some important metabolites. Then the quality of the finished beers was evaluated.

<sup>1</sup> This study was carried out in Laboratoire de Génie Chimique UMR-CNRS 5503, 18 Chemin de la Loge, 31078 Toulouse Cedex 4, France.

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## EXPERIMENTAL

### Media

Four different worts were used. W1 was wort from 60% malt and 40% rice. W2 was wort from 60% malt, 40% rice, and Neutrase 0.5L (Novo-Nordisk, Bagsvaerd, Denmark), containing a neutral protease. According to the recommended guidelines, the enzyme dose employed was 0.07% of brewing materials. W3 was wort from 60% malt, 40% rice, and yeast extract (Biokar, Beauvais, France) added at 2g/L. W4 was wort from 100% malt.

Media W1, W2, and W3 were prepared by decoction mashing. Twenty five percent of the malt used for each mashing was added to the adjunct mash for rice liquefaction. Adjunct-mashing was carried out at 70°C for 45 min, and then at 100°C for 30 min. Malt-mashing began at 50°C; the malt-mash was then mixed with the adjunct-mash. The mashing process was continued at 63°C for 15 min and at 70°C until total saccharification (checked by the I<sub>2</sub> test).

Medium W4 was prepared by infusion mashing. Three stages were effected at 50, 63, and 70°C.

Neutrase 0.5L, was added in the malt-mash for medium W2. The proteolytic stand was carried out at 50°C for 60 min in all mashes. Mash pH values were not regulated. Wort boiling with hop addition was carried out at 100°C for 1 hr. Then the centrifuged wort was adjusted to 11°P specific gravity and sterilized at 121°C for 20 min in the fermentors. Yeast extract was dissolved in ultrapure water to 11% mass solution and sterilized at 121°C for 15 min. It was then added to the fermentor for medium W3. Finally, the worts were aseptically aerated with up to 7 ppm dissolved oxygen (using a 0.2- $\mu$ m membrane filter; Sartorius AG, Goettingen, Germany).

In this study, malt was supplied by Danone Society (Strasbourg, France), rice by Siam Ka Kao Co., Ltd. (Bangkok, Thailand), and hop extract ( $\alpha$ -acids content: 56.8% dry weight) by Joh. Barth & Sohn Nurnberg (Nurnberg, Germany).

### Yeast

The strain *Saccharomyces cerevisiae* Nottingham, commercialized by Lallemand Inc. as "active dried yeast," was used. Although the recommended temperature for fermentation using this yeast is quite high (14–21°C), Nottingham is a flocculant strain. To stabilize the inoculum activity for repetition during this study, the powdered yeast was first activated in the malt wort. The cells were then maintained on malt agar medium (Diagnostic-Pasteur, Marnes-la-Coquette, France). The strain was stored at 4–6°C and transferred onto new malt agar medium every three months. The inocula were prepared from these tubes.

### Inoculum

Precultures were prepared by two successive inoculations: 1) in a 250-ml Erlenmeyer shake-flask containing 100 ml of 11°P malt wort for 14 hr, and 2) in a 2-L Erlenmeyer shake-flask containing 500 ml of 11°P malt wort for 10 hr. For both periods, the inoculum was grown at 28°C and 250 rpm. The pitching rate was  $3 \times 10^6$  viable cells per milliliter.

### Fermentation

The fermentations were made at 14°C in four 2.5-L fermentors, each with a 2-L working volume, containing the four media described above. The fermentors were immersed in a water bath connected with a cooling apparatus (B. Braun Biotech. International, Melsungen, Germany). The homogeneity of the media was achieved by magnetic agitation (100 rpm, magnetic stirrer size: 8  $\times$  45 mm).

All fermentations were conducted in duplicate.

### Maturation

At the end of fermentation, magnetic agitation was stopped. The media were maintained in the same fermentors at 8°C for 24 hr for yeast flocculation to occur. The green beer was then transferred to 250-ml bottles sealed by a metallic capsule. The maturation was carried out at 4°C for 15 days.

### Analytical Methods

Yeast growth was determined as dry weight, using a cellulose acetate membrane with a pore size of 0.45  $\mu$ m (Sartorius AG). Yeast viability was analyzed by hemocytometry using the methylene blue test.

Specific gravity, free amino nitrogen, and vicinal diketones of fermenting wort were determined by densimeter, ninhydrin, and spectrophotometric methods, respectively, according to recommended methods of the European Brewery Convention (10).

Ammonium nitrogen was analyzed by a spectrophotometric method with blue indophenol (Norme Francaise NF T90-015) (2).

Proteins were measured by the Bradford method, using bovine albumin (Sigma Chemical Co., St. Louis, MO) as the standard protein.

Fraction C nitrogen (Lundin) was determined by precipitating fractions A and B in wort using sodium molybdate and sulfuric acid at 20°C, eliminating precipitate by filtration, and determining total nitrogen in the filtrate. The analytical procedure is detailed elsewhere (20).

For total nitrogen determination, the sample was digested by H<sub>2</sub>SO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> at 440°C for mineralization (Digesdalh, Hach, Loveland, CO). The nitrogen concentration was subsequently analyzed by the Nessler method (reactive of Fluka Co., New-Ulm, Switzerland).

Ethanol was analyzed by gas chromatography (Chrompack, Delft, The Netherlands) using a wide-bore column (0.53 mm  $\times$  25 m, Chrompack Poraplot Q) combined with an integrator SP 4290 (Spectra-Physics, San Jose, CA). An 1% (v/v) isopropanol solution was used as the internal standard.

Glycerol and acetic acid were determined by enzymatic methods using kits 148270 and 148261, respectively (Boehringer Mannheim GmbH, Mannheim, Germany).

Pyruvic acid was analyzed by an enzymatic method (Boehringer Mannheim GmbH).

Dissolved oxygen was determined by a sterilizable O<sub>2</sub> sensor (Mettler Toledo, Paris, France).

Sensory analysis was conducted according to method NF ISO 8587, reported elsewhere (1). Beer flavor was described with international beer flavor terminology (22).

Amino acids were determined in Laboratoire du Departement Biochimie Alimentaire INSA, Toulouse, by an HPLC method (Hewlett-Packard, Les Ulis, France), using system AMINOQUANT HP 1090, which employs a precolumn for amino acid derivatization. Primary amino acids were modified by ortho-phthaldehyde (OPA) and secondary ones by 9-fluorenylmethyl chloroformate (FMOC). The complexes were then separated according to their hydrophobicity by a specific amino acids column C18 (ref. 79916 AA-572 HP, Hewlett-Packard). Detection was carried out by a spectrophotometer with a diodic bar at two wavelengths: 338 nm for OPA complexes and 262 nm for FMOC complexes.

## RESULTS AND DISCUSSION

### Analysis of the Media

Some characteristics of the media are presented in Table I. Utilization of 40% rice considerably decreased the concentration of nitrogen compounds in the wort. The ammonium nitrogen and free amino nitrogen concentrations in medium W 1 were about 80 and 60%, respectively, of those in malt medium W4.

Adding microbial protease in the malt-mash or supplementing the wort with yeast extract significantly improved the nitrogen composition of the medium. In medium W2, Neutrase 0.5L raised the quantity of the total nitrogen, but its concentration was less than for medium W4. An increase of free amino nitrogen was noted, but it remained far lower than that in malt medium W4. Neutrase 0.5L did not influence the ammonium nitrogen level. The increase of fraction C nitrogen suggests that the neutral protease of Neutrase 0.5L catalyzes mainly the liberation of certain polypeptides and peptides during mashing.

Yeast extract (medium W3) considerably increased both the total nitrogen and the free amino nitrogen content in the wort. The concentrations were similar to those in malt medium W4. Furthermore, levels of fraction C nitrogen were raised because of the peptides and polypeptides present in the yeast extract. There was a negligible increase in ammonium nitrogen.

In summary, adding protease during mashing leads principally to polypeptide accumulation and increases the total nitrogen in wort, whereas addition of yeast extract increases the free amino nitrogen content.

### Yeast Growth

Figure 1 shows the kinetics of yeast growth during fermentation. Biomass formation was similar on media W3 and W4. The maximum concentration of biomass obtained was about 4.2 g/L after 72 hr of incubation. On medium W2, the logarithmic phase continued until 96 hr. However, the biomass yield reached almost the same value as those on media W3 and W4. On medium W1, yeast growth was slow. The maximum biomass concentration (2.9 g/L) was obtained after 120 hr.

TABLE I  
Some Physicochemical Characteristics of the Media<sup>a</sup>

Characteristic	W1	W2	W3	W4
Specific gravity, P	11.0	11.0	11.0	11.0
Ammonium nitrogen (N, mg/L)	20	20	21	26
Free amino-nitrogen (N, mg/L)	86	107	160	146
Fraction C nitrogen (N, mg/L)	225	320	325	369
Total nitrogen (N, mg/L)	453	558	599	625
pH	5.20	5.20	5.39	5.34

<sup>a</sup> W1 = wort from 60% malt and 40% rice. W2 = wort from 60% malt and 40% rice with added Neutrase. W3 = wort from 60% malt, 40% rice, and yeast extract. W4 = wort from 100% malt.

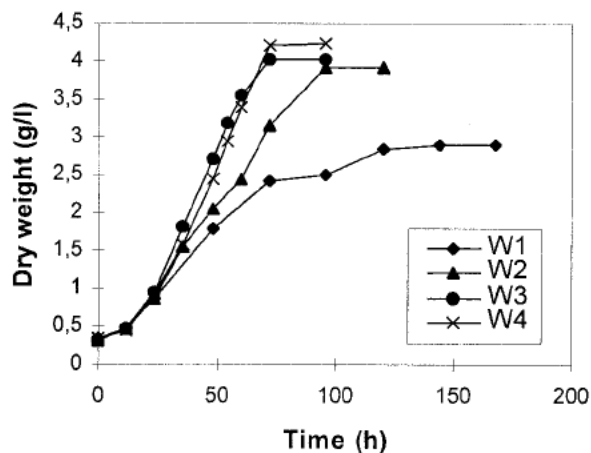


Fig. 1. Kinetics of yeast growth. W1 = wort from 60% malt and 40% rice, W2 = wort from 60% malt and 40% rice with added Neutrase, W3 = wort from 60% malt, 40% rice, and yeast extract, W4 = wort from 100% malt.

Difference in kinetics and biomass yield of yeast growth on the four media with the same specific gravity may be due to the difference in the amount of assimilable nitrogen and microelements. It is evident that the carbon source is not responsible for the growth difference in this study. Some authors affirmed that yeast growth is governed to a large extent by the source and the level of assimilable nitrogen in wort (4,30).

With regard to yeast viability, the culture populations were generally observed to have a good physiological state during fermentation on the four media. Dead cells did not exceed 5%.

### Substrate Assimilation

Density analysis is given in Figure 2. The fermentation was similar and rapid on media W3 and W4, lasting for 96 hr. For medium W2, the fermentation time was about 120 hr. Fermentation was very slow on medium W1, consistent with the decrease of yeast growth. Residual sugars were highest for this medium (data not shown). It appears that the rate of sugar assimilation by yeast is reduced in the latter stages of fermentation, perhaps due to low metabolic activity of the culture population.

Sugar transformation in alcoholic fermentation is closely related to yeast growth (reviewed by Devreux [7]). In this study, supplementation with 40% rice doubled the fermentation time in comparison with that of the 100% malt. This would lead to a considerable decrease of fermentor capacity on an industrial scale. Yeast extract is better than microbial protease for acceleration of fermentation.

The kinetics of nitrogen compound utilization are shown in Figures 3–6. Ammonium nitrogen was assimilated by yeast during the early stages of fermentation. Figure 3 shows the complete disappearance of this compound for all media after 36–48 hr. Jones and Pierce proposed the classification of amino acids and ammonia according to their speed of absorption from wort under brewing conditions (16). In this classification, ammonia belongs to group C, wherein a nitrogen compound is assimilated by yeast only after 20–24 hr, coinciding with the complete elimination of group A amino acids from the medium (16). Similar results were observed by different studies with both bottom- (21,29) and top-fermenting brewing strains (30). However, Devreux claimed that the reason for this sequential utilization of amino acids and ammonia by brewing yeast was not clear (7). Dubois and Greson (8) proposed a mechanism in which the ammonia transport was realized by accumulative electrophoretic single port. The transport

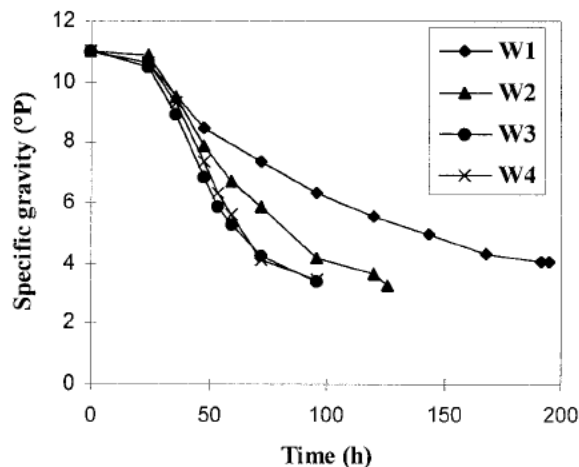


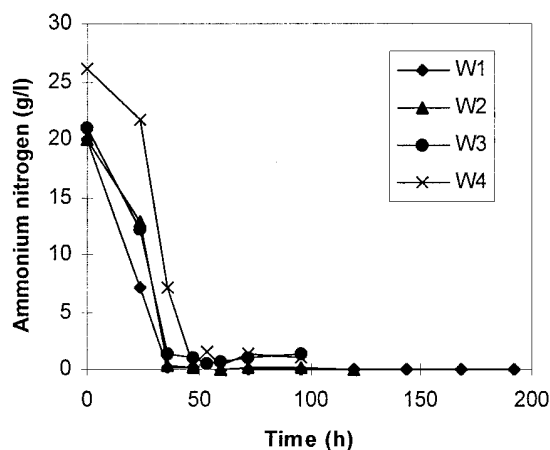
Fig. 2. Kinetics of wort specific gravity, W1 = wort from 60% malt and 40% rice, W2 = wort from 60% malt and 40% rice with added Neutrase, W3 = wort from 60% malt, 40% rice, and yeast extract, W4 = wort from 100% malt.

activity may be noncompetitively inhibited by certain amino acids, particularly arginine and aspartic acid (9). These amino acids belong to group A.

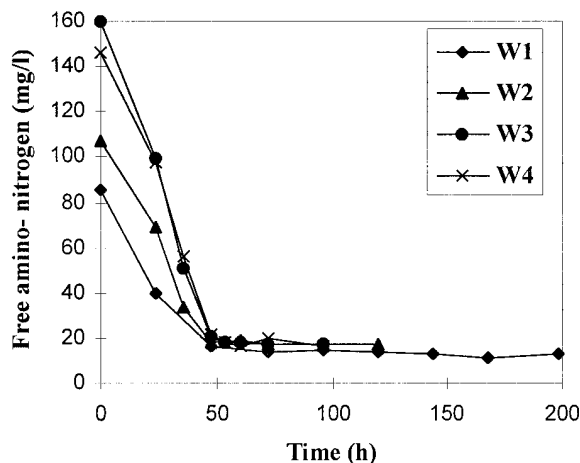
In this study, after the first 24 hr, some ammonium nitrogen was already consumed in all four media. This observation differs from those previously observed in the literature. However, similar results were shown with wine strains. According to Jiranek et al (15), in such strains, ammonia belongs to group A. This group is absorbed rapidly and almost linearly from the medium, resulting in its elimination early in the growth cycle.

Note that during the first 24 hr, assimilated ammonium nitrogen from medium W1 was higher than that in media W2, W3, and W4. As Egbosimba and Slaughter (9) concluded, perhaps the rapid exhaustion of some amino acids of group A such as arginine and aspartic acid, which have lower concentrations in W1 than in W2–W4 (Table II), leads to the increase in ammonia assimilation during this period.

Nevertheless, the capacity for absorption of ammonium nitrogen by the Nottingham strain during the first period of fermentation is quite clear. It seems that this yeast's priority for absorption of nitrogen compounds is the same as for absorption of wine strains.



**Fig. 3.** Consumption of ammonium nitrogen during fermentation, W1 = wort from 60% malt and 40% rice, W2 = wort from 60% malt and 40% rice with added Neutrased, W3 = wort from 60% malt, 40% rice, and yeast extract, W4 = wort from 100% malt.



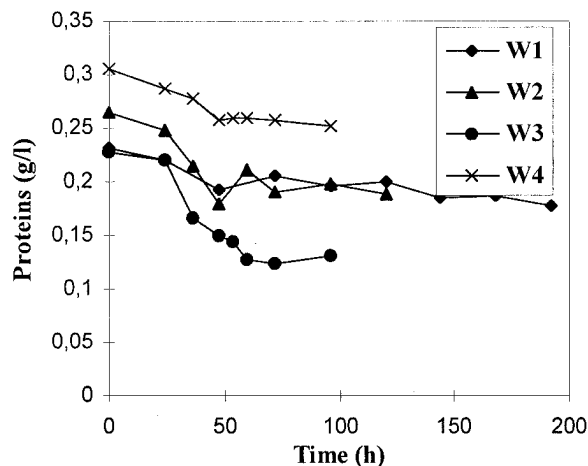
**Fig. 4.** Consumption of free amino-nitrogen (ninhydrin method) during fermentation, W1 = wort from 60% malt and 40% rice, W2 = wort from 60% malt and 40% rice with added Neutrased, W3 = wort from 60% malt, 40% rice, and yeast extract, W4 = wort from 100% malt.

After 48 hr, the level of free amino nitrogen consumed was the same for all four media (Fig. 4). As biomass formation continued until 72–120 hr, the question is: after 48 hr, which nitrogen source did the yeast consume for growth?

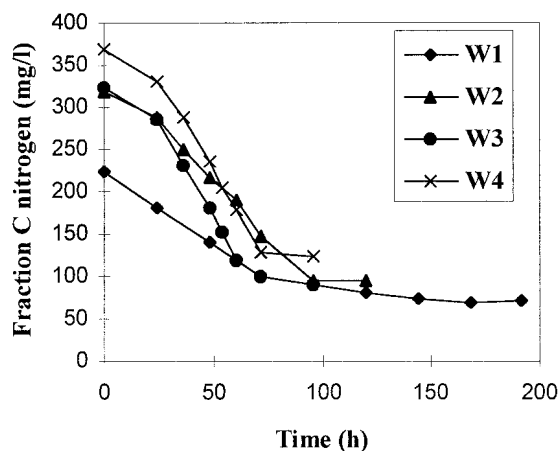
Figure 5 indicates that protein concentration decreased during fermentation, partly due to precipitation of some proteins by pH change. On the other hand, protease activity was similar in the four media during fermentation (data not shown). It is supposed that the protease was secreted by yeast (28). This protease can presumably hydrolyze certain proteins and polypeptides in wort in order to supply assimilable nitrogen to support yeast growth after 48 hr of incubation. However, the protease activity is quite low, and the assimilable nitrogen liberated through this proteolysis may be insufficient to contribute to biomass formation.

The ninhydrin method gives an estimate of amino acids, ammonia, and also the terminal  $\alpha$ -amino nitrogen groups of peptides and proteins (10). It is likely that this method does not accurately reflect the quantity of assimilated nitrogen compounds in wort during fermentation.

Figure 6 shows a decrease in fraction C nitrogen concentration during the logarithmic phase of yeast growth on all media. This



**Fig. 5.** Changes in protein concentration in the four media during fermentation, W1 = wort from 60% malt and 40% rice, W2 = wort from 60% malt and 40% rice with added Neutrased, W3 = wort from 60% malt, 40% rice, and yeast extract, W4 = wort from 100% malt.



**Fig. 6.** Kinetics of fraction C nitrogen assimilation during fermentation, W1 = wort from 60% malt and 40% rice, W2 = wort from 60% malt and 40% rice with added Neutrased, W3 = wort from 60% malt, 40% rice, and yeast extract, W4 = wort from 100% malt.



TABLE II  
Amino Acid Analysis of the Media<sup>a</sup>

Amino Acid Concentration, mg/L	Medium W1		Medium W2		Medium W3		Medium W4	
	(1) <sup>b</sup>	(2)	(1)	(2)	(1)	(2)	(1)	(2)
ASP	30.7	0	35.1	0	71.3	0	55.0	0
GLU	31.6	8.5	39.2	7.6	193.5	10.2	47.1	7.8
SER	29.3	0	41.4	0	68.3	0	58.5	0
HIS	17.3	0	28.0	0	33.7	0	41.6	0
GLY	18.6	0	25.2	0	44.9	0	36.1	0
THR	26.6	0	42.5	0	66.1	0	56.2	0
ALA	51.6	3.4	76.0	4.2	131.1	3.7	101.4	0
ARG	85.0	0	125.5	0	125.7	0	181.1	0
TYR	38.7	0	69.7	0	59.1	0	84.6	0
CYS	17.3	0	48.1	0	43.1	0	62.7	0
VAL	44.3	0	70.7	0	100.5	0	93.6	0
MET	18.3	3.3	34.7	6.6	35.7	6.9	47.1	7.3
PHE	45.8	0	73.5	0	94.7	0	91.8	0
ILE	28.9	0	46.5	0	78.5	0	59.0	0
LEU	59.5	0	108.6	0	149.3	0	125.8	0
LYS	32.8	0	60.3	0	80.1	0	70.6	0
PRO	163.4	35.3	170.1	39.1	245.2	37.1	231.8	25.9

<sup>a</sup> W1 = wort from 60% malt and 40% rice. W2 = wort from 60% malt and 40% rice with added Neutrase. W3 = wort from 60% malt, 40% rice, and yeast extract. W4 = wort from 100% malt.

<sup>b</sup> 1 = wort at the beginning of fermentation, 2 = wort at the end of fermentation.

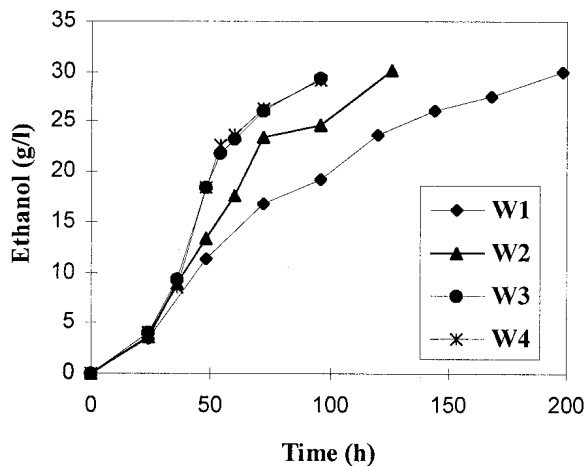


Fig. 7. Kinetics of ethanol formation in the four media, W1 = wort from 60% malt and 40% rice, W2 = wort from 60% malt and 40% rice with added Neutrase, W3 = wort from 60% malt, 40% rice, and yeast extract, W4 = wort from 100% malt.

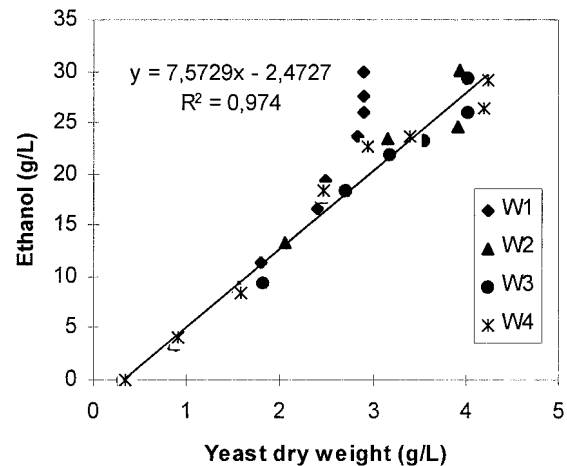


Fig. 8. Relationship between yeast growth and ethanol formation in the four media, W1 = wort from 60% malt and 40% rice, W2 = wort from 60% malt and 40% rice with added Neutrase, W3 = wort from 60% malt, 40% rice, and yeast extract, W4 = wort from 100% malt.

decrease correlates with the kinetics of biomass formation. The levels of fraction C nitrogen remain in the range of 72–124 mg/L in the four media at the end of the fermentation, because certain polypeptides are not assimilated by yeast. A low assimilable nitrogen concentration in medium W1 retards biomass formation.

Amino acid concentrations in the four media are summarized in Table II. Amino acids in all four media were exhausted at the end of the fermentation. Proline was partially assimilated by this yeast strain. Many studies have shown that proline is not utilized by yeasts under brewing conditions (21,31). Although it is a major component of malt wort (31) and fermented grape juice (11), it has no free amino group and therefore cannot take part directly in transamination reactions. According to Wang and Brandiss (33), proline does not serve as a nitrogen source under anaerobic conditions because the proline oxidase gene, *PUTI*, is located in the mitochondria. Proline oxidase is a mitochondrial plasma membrane-associated enzyme that requires a functional electron transport chain and aerobiosis for its activity.

In a study on wine fermentation, Ingledew et al showed that the provision of small but adequate amounts of oxygen can lead to

virtually complete proline utilization by a strain of wine yeast (13). However, the agitation achieved by the stirring system employed in this study was too low to support aeration of 2 L of medium in the 2.5-L fermentor.

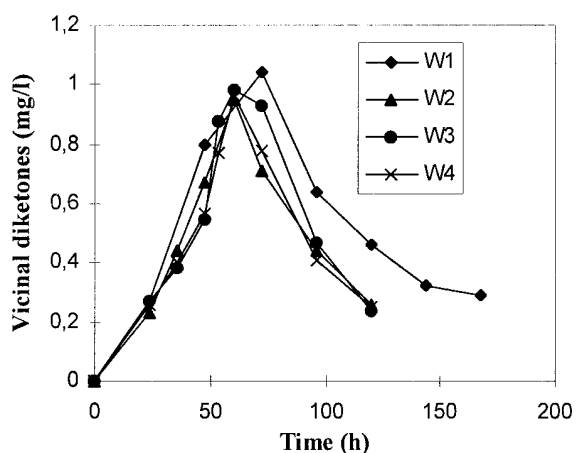
#### Metabolite Production

The kinetics of ethanol formation are given in Figure 7. Ethanol production is very slow on medium W1 in comparison with that on media W2–W4. The use of yeast extract and microbial protease promoted ethanol production in malt-rice brewing.

Figure 8 illustrates the relationship between yeast biomass formation and ethanol production for all four media. The same linear correlation between yeast growth and ethanol production was demonstrated for media W2–W4. However, this correlation was clearly modified for medium W1.

Glycerol is a principal by-product of alcoholic fermentation related to the multiplication of biomass or the enhancement of the ethanol content (17). Its concentration was about 2.2–2.5 g/L for the four media at the end of fermentation.

During the fermentation, a notable decrease of pH was observed



**Fig. 9.** Vicinal diketone formation during fermentation, W1 = wort from 60% malt and 40% rice, W2 = wort from 60% malt and 40% rice with added Neutrase, W3 = wort from 60% malt, 40% rice, and yeast extract, W4 = wort from 100% malt.

on the four media. At the end of fermentation, the pH values of media W1, W2, W3, and W4 were 3.60, 3.49, 3.79, and 3.74, respectively.

According to Coote and Kirsop (6), fermentation with yeast propagated under semiaerobic conditions rather than in anaerobic fermentors gave beers with lower pH and increased organic acid content, but the latter factor was not in itself sufficient to account for the pH difference. These authors showed that the pH values fell to 3.7–3.9 on all malt-hopped wort for some brewing yeast strains propagated in shaken Roux bottles with air present in the headspace of the bottle. It was also reported that organic acid excretion and absorption of basic amino acids both have substantial effects on pH decrease during fermentation. On the other hand, dissolution of carbon dioxide and absorption of phosphate contribute to a small extent.

In this study, even on the malt medium (W4), the pH obtained was about 3.8. The considerable assimilation of basic amino acids by Nottingham strain yeast during fermentation (Table II) partially explains the pH drop. Additionally, it seems that this strain produces a significant quantity of organic acids. Finally, the preparation of inoculum in “semi-aerobic conditions” may explain the pH decrease. However, the pH difference of the four media at the end of fermentation is not very significant ( $\Delta\text{pH} = 0.3$ ).

Figure 9 shows that vicinal diketone removal in medium W1 is slower than that for the three other fermentations.

### Product Analysis

Analysis of the beers produced in these studies is given in Table III.

There are no substantial differences between the four beer samples, except in their vicinal diketone content. The quantity of vicinal diketones in beer sample W1 is obviously higher than in the other three samples. The formation and elimination of vicinal diketones are affected by the soluble nitrogen level and the spectrum of amino acids in the wort (27,31). According to Miedaner et al (24), low amino nitrogen levels tend to lead to high levels of diacetyl during fermentation. Hoffmann (12) affirmed that wort amino nitrogen should be greater than 200 mg/L to ensure that the diacetyl level in the final beer is less than 0.12 mg/L.

The content of organic acids in the four beer samples is not high and does not overrun the threshold recommended elsewhere (25).

Sensory analysis of the beers was performed with 11 subjects according to a “rank classification” method (1). Four blind samples were classified by the subjects according to preference terms.

**TABLE III**  
Physicochemical Analysis of Finished Beer<sup>a</sup>

Characteristic	Sample W1	Sample W2	Sample W3	Sample W4
Apparent extract P	4.0	3.45	3.4	3.4
Ethanol, g/L	30.6	32.9	33.5	33.8
Vicinal diketones, mg/L	0.26	0.14	0.14	0.15
Acetic acid, mg/L	24.2	19.5	31.9	26.1
Pyruvic acid, mg/L	48.9	60.1	96.5	92.1
pH	3.68	3.59	3.83	3.84

<sup>a</sup> W1 = wort from 60% malt and 40% rice. W2 = wort from 60% malt and 40% rice with added Neutrase. W3 = wort from 60% malt, 40% rice, and yeast extract. W4 = wort from 100% malt.

**TABLE IV**  
Sensory Analysis of Beer Flavor by the Rank Classification Method

Subject	Beer Flavor Rank			
	W1 <sup>a</sup>	W2	W3	W4
1	3	4	2	1
2	4	2	3	1
3	4	1	3	2
4	3	4	1	2
5	4	3	2	1
6	4	1	3	2
7	3	4	1	2
8	4	2	3	1
9	3	4	1	2
10	4	3	1	2
11	4	3	2	1
Sum of ranks	40	31	22	17

<sup>a</sup> W1 = wort from 60% malt and 40% rice. W2 = wort from 60% malt and 40% rice with added Neutrase. W3 = wort from 60% malt, 40% rice, and yeast extract. W4 = wort from 100% malt.

Ranks of 1–4 were attributed to the beers, with 1 the most and 4 the least preferred (Table IV).

The preferred beer was the sample from 100% malt (W4), with W3 being second. The flavor of this sample was similar to that of sample W4.

Although the physicochemical characteristics of sample W2 were similar to those of sample W4, its organoleptic quality was deemed unacceptable. The taste and the odor of this sample were preferred to those of sample W1, but the beer was “watery.” Sample W1 was rated diacetyl and watery with a characterless mouthfeel.

The statistical treatment of the results was calculated by the Friedman criterion (31):

$$F = \left[ \frac{12}{J \times P \times (P+1)} (R_1^2 + R_2^2 + \dots + R_p^2) \right] - 3J(P+1)$$

where  $J$  is the number of subjects ( $J = 11$ ),  $P$  is the number of samples ( $P = 4$ ), and  $R_1, R_2, \dots, R_p$  equal the sum of the ranks attributed by each one of the  $J$  subjects for each sample. Therefore,

$$F = \left[ \frac{12}{11 \times 4 \times (4+1)} (40^2 + 31^2 + 22^2 + 17^2) \right] - 3 \times 11 \times (4+1) = 16.8$$

The calculated value  $F_{\text{calculated}} = 16.8$  was superior to the critical one ( $F_{\text{critical}} = 7.81$ ) on the signification threshold  $\alpha = 0.05$  (1). The four beer samples were confirmed to be different with 5% maximum error risk.

### CONCLUSION

Use of 40% rice in the grist presents substantial technological difficulties. The decrease of assimilable nitrogen in the wort slows down yeast growth and increases both fermentation time and

residual sugar content in the beer. Product quality is affected, particularly the organoleptic characteristics.

Adding protease to the mash and supplementing boiled wort with yeast extract are possible solutions to overcome the nitrogen limitation. Supplementing with yeast extract gives better results than adding protease, not only for fermentation performance but also for beer quality. It is, however, a costly option.

#### LITERATURE CITED

1. Association Francaise de Normalisation (AFNOR). *Recueil de Normes Françaises 1995, Contrôle de la Qualité des Produits Alimentaires. Analyse Sensorielle*, 5th ed. The Association, Paris, 1995.
2. Association Francaise de Normalisation (AFNOR). *Norme Française—La Qualité de L'eau*, Vol. 3. The Association, Paris, 1999.
3. Bilinski, A. A., and Stewart, G. G. Yeast proteases and brewing. Pages 147-162 in: *Yeast: Biotechnology and Biocatalysis*, Vol. 5. H. Verachert and R. De Mot, eds. Marcel Dekker Inc., New York, 1990.
4. Boulton, C. A. Development in brewing fermentation. Pages 127-182 in: *Biotechnology and Genetic Engineering Reviews*, Vol. 9. M. B. Tombs, ed. Intercept Ltd, Andover, U.K., 1991.
5. Connor-Cox, E. S. C., and Ingledew, W. M. Wort nitrogenous sources—Their use by brewing yeasts: A review. *J. Am. Soc. Brew. Chem.* 47:102-108, 1989.
6. Coote, N., and Kirsop, B. H. Factors responsible for the decrease in pH during beer fermentations. *J. Inst. Brew.* 82:149-153, 1976.
7. Devreux, A. Les acides aminés, modulateurs du goût de la bière. *Cerevisia* 10:59-70, 1985.
8. Dubois, E., and Grenson, M. Methylamine/ammonia uptake systems in *Saccharomyces cerevisiae*: Multiplicity and regulation. *Mol. Gen. Genet.* 175:67-76, 1979.
9. Egbosimba, E. E., and Slaughter, J. C. The influence of ammonium permease activity and carbon source on the uptake of ammonium from simple defined media by *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* 133:375-379, 1987.
10. European Brewery Convention. *Analytica EBC*, 4th ed. Brauerei- and Getränke-Rundschau, Zurich, 1987.
11. Henschke, P. A., and Jiranek, V. Yeasts—Metabolism of nitrogen compounds. Pages 77-164 in: *Wine Microbiology and Biotechnology*. G. H. Fleet, ed. Harwood Academic Publishers, Singapore, 1993.
12. Hoffmann, S. Formation and degradation of diacetyl. *Brauwelt Int.* 1:59-60, 1985.
13. Ingledew, W. M., Magnus, C. A., and Sosulski, F. W. Influence of oxygen on proline utilization during the wine fermentation. *Am. J. Enol. Vitic.* 38:246-248, 1987.
14. Inoue, T. The relationship between the performance of yeast and acetohydroxy acid formation during wort fermentation. *Tech. Q. Master Brew. Assoc. Am.* 17(2):62-65, 1981.
15. Jiranek, V., Langridge, P., and Henschke, P. A. Nitrogen requirement of yeast during wine fermentation. Pages 166-171 in: *Proc. Australian Wine Industry Technical Conference, 7th*. P. J. Williams, D. M. Davidson, and T. H. Lee, eds. Australian Industrial Publishers SA, Adelaide, 1990.
16. Jones, M., and Pierce, J. S. Absorption of amino acids from wort by yeasts. *J. Inst. Brew.* 70:307-315, 1964.
17. Klopfer, W. J., Angelino, S. A. G. F., Tuning, B., and Vermeire, H. A. Centenary review: Organic acids and glycerol in beer. *J. Inst. Brew.* 92:225-228, 1986.
18. Lloyd, W. J. W. Centenary review: Adjuncts. *J. Inst. Brew.* 92:336-345, 1986.
19. MacDonald, J., Reeve, F. T. V., Ruddlesden, J. D., and White, F. H. Current approaches to brewing fermentations. *Prog. Indust. Microbiol.* 19:1-197, 1984.
20. Malsev, P. M., Velikaia, E. I., Zazirnaia, M. V., and Kolotusha, P. V. *Chemico-Technological Control in Malting and Brewing*. Pisch. Prom-st, Moscow, 1976.
21. Marc, I. Modélisation de la fermentation de la bière dans un réacteur cylindro-conique. Thèse docteur ingénieur, Institut National Polytechnique de Lorraine, Nancy, France, 1982.
22. Meilgaard, M. C. Beer flavour terminology. *J. Inst. Brew.* 85:38-42, 1979.
23. Melejev, A. Y., and Nguyen, H. T. Intensification of maize and rice proteolysis in brewing. *Izv. Vyssh. Uchebn. Zaved. Pishch. Tekhnol.* (3-4):17-20, 1992.
24. Miedaner, H., Kunz, A., and Narziss, L. The effect of free amino nitrogen on fermentation and the first stages of production of vicinal diketones. *Proc. Congr. Eur. Brew. Conv.* 17:725-735, 1979.
25. Moll, M. *Bières et Coolers*. Tec & Doc, Lavoisier, Paris, 1991.
26. Moll, M., Flayeux, R., Vinh, T., and Martin, J. Composition azotée des moût destinés a la fermentation basse. *Bios* 5:457-474, 1974.
27. Narziss, L., Miedaner, H., and Gresser, A. Yeast races and beer quality. Formation and reduction of vicinal diketones and their precursors as well as acetoin during fermentation. *Brauwelt* 124:494-497, 500-501, 1984.
28. Ormrod, I. H. L., Lalor, E. F., and Sharpe, F. R. Yeast proteolytic enzyme activity during fermentation. *Proc. Congr. Eur. Brew. Conv.* 23:457-464, 1991.
29. Palmqvist, U., and Äyräpää, T. Uptake of amino acids in bottom fermentations. *J. Inst. Brew.* 75:181-190, 1969.
30. Pierce, J. S. The Margaret Jones Memorial Lecture: Amino acids in malting and brewing. *J. Inst. Brew.* 88:228-233, 1982.
31. Pierce, J. S. Adjuncts and their effects on beer quality. *Proc. Congr. Eur. Brew. Conv.* 21:49-60, 1987.
32. Suomalainen, H., and Oura, E. Yeast nutrition and solute uptake. Pages 3-74 in: *The Yeasts*, Vol. 2. A. H. Rose and J. S. Harrison, eds. Academic Press, London., 1971.
33. Wang, S. S., and Brandiss, M. C. Proline utilization in *Saccharomyces cerevisiae*: Sequence, regulation and mitochondrial localization of the PUT I gene product. *Mol. Cell. Biol.* 7:4431-4440, 1987.
34. Yoshioka, K., and Hashimoto, N. Change in the composition of wort during the mashing process. *Tech. Q. Master Brew. Assoc. Am.* 16:82-89, 1979.