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Uptake of Amino Acids During Beer Production: The Concept of a Critical Time Value

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ABSTRACT

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According to M. Jones and J. Pierce in 1964, amino acid uptake occurs sequentially when *Saccharomyces cerevisiae* grows in a complex medium, whatever the individual concentration. The objective of the current work was to determine to what extent, under different conditions, the four previously defined amino acid families can still be distinguished. For this purpose, amino acid uptake was monitored during fermentation in the presence of one ale and two lager yeasts conducted at various scales (1-L tall EBC tubes and 1-L conical flasks) and temperatures (23 and 28°C). Industrial production (1-hL rectangular vessel) conducted with a co-culture of three ale yeasts was also investigated. A critical time (Tc), defined as the time it takes the amino acids of group A' (named A' in the current work) to be totally consumed, emerged from all our experiments. This group coincides with the A class defined by M. Jones (aspartate, threonine, serine, glutamate, lysine, and arginine) plus methionine and minus arginine. Tc also corresponds with the beginning of consumption of an amino acid group (named C' in the current work) that includes only glycine and alanine. All other amino acids, defining the B' group, (named B' in the current work) are slowly and gradually taken up without any lag phase.

Keywords: Fermentation, Permeases, *Saccharomyces cerevisiae*

RESUMEN

Consumo de Aminoácidos Durante Producción de Cerveza: El Concepto de un Valor Crítico de Tiempo

Según M. Jones y J. Pierce en 1964, el consumo de aminoácido ocurre secuencialmente cuando *Saccharomyces cerevisiae* crece en un medio complejo, cual sea la concentración individual. El objetivo del trabajo actual era determinar a qué medida, bajo diversas condiciones, las cuatro familias previamente definidas de aminoácidos todavía pueden ser distinguidas. Para este propósito, el consumo de aminoácido se superviso durante fermentación en presencia de una levadura ale y dos levaduras lager conducidas en varias escalas (tubos altos de 1-L EBC y frascos cónicos de 1-L) y temperaturas (23 y 28°C). Una producción industrial (recipiente rectangular de 1-hL) conducida con co-cultura de tres levaduras ale también fue investigada. Un tiempo crítico (Tc), definido como el tiempo que se toman los aminoácidos del grupo A' (nombrado A' en el trabajo actual) para ser totalmente consumidos, se dio en todos nuestros experimentos. Este grupo coincide con la clase A definida por M. Jones "(aspartate, threonine, serine, glutamate, lysine, y arginine)" más "methionine" y menos "arginine". El Tc también corresponde con el principio de consumición de un grupo de aminoácidos (nombrado C' en el trabajo actual) que incluye solamente "glycine" y "alanine". El resto de los aminoácidos, definido grupo B', (nombrado B' en el trabajo actual) lenta y gradualmente fueron consumidos sin ninguna retraso de fase.

Palabras claves: Fermentación, Permeasa, *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae takes up amino acids from the wort. After Ehrlich degradation, some are converted to metabolites such as alcohols and esters, imparting the typical fruity flavors to beer (4). Control of these favorable aromas could be improved by understanding how amino acids are taken up from a complex medium such as wort.

Unfortunately, most of the available data concern laboratory strains in model media that often contain only one nitrogen source. Amino acids are known to be transported into the yeast cell by permeases of the AAP (amino acid permease) family, which includes 18 members (1). The family also includes a unique member called Ssy1p (6,7), differing from the others by the low expression level of its gene and an unusually long N-terminal domain, essential to its function as a sensor (10). Overproduction of the N-terminal domain interferes with Ssy1p function in a dominant-negative manner (2,8). Genes such as *AGP1*, *BAP2*, *BAP3, TAT1,* or *TAT2* have been shown to require Ssy1p for their expression (5,8). According to Forsberg et al (7), Ssy1p, Ptr3p, and Ssy5p are components of a sensor complex (SPS) localized in the plasma membrane and have recently emerged as being involved in the control of a large number of other permeases (11).

Since Ssy1p was revealed as crucial for amino acid uptake in a complex medium such as wort (13), we can expect that Ssy1p-dependent permeases are expressed and are responsible for the sequence of amino acid entering into the cell. According to Regenberg et al (16), the majority of the amino acids could enter the cell as soon as these transporters are functional. However, Thorne (17) has shown how individual amino acid, when used as the sole source of nitrogen, are absorbed from a synthetic medium and how mutual antagonism and synergism may occur when a mixture of amino acids are present in the medium.

In a complex media such as wort, brewers usually distinguish four groups of amino acids (9,12,14). In the usual model, Group A, which includes aspartate, threonine, serine, glutamate, lysine, and arginine, is reported to be immediately absorbed and almost totally consumed after a few hours of fermentation. Group B, which includes valine, methionine, isoleucine, leucine, and histidine, is said not to be removed rapidly, but absorbed gradually throughout fermentation. Alanine, tyrosine, phenylalanine, tryptophane, and glycine define group C, which is characterized by a very long lag phase. These amino acids are used only when group A is totally depleted. Proline, finally, is known to be only slightly absorbed from wort under anaerobic conditions.

Of course, such a classification depends on the criteria used, either the time required to reach half concentration or the initial removal rate. Therefore, amino acids such as methionine (15) or glutamate and aspartate (12) have been sometimes removed from one group to the other to better fit the experimental data.

The objective of the current work was to refine this model by introducing a new factor and to check the validity of this parameter while varying experimental conditions such as temperature, strain, and vessel type. A comparison with an industrial fermentation was also included.

EXPERIMENTAL

Yeast Strains

Saccharomyces cerevisiae strains BRAS291, BRAS12 (bottom fermentation yeasts), and BRAS212 (top fermentation yeast) were provided by the BRAS collection of the Université catholique de Louvain, Louvain-la-Neuve, Belgium. Three industrial strains (top fermentation yeasts) were provided by the Trappist Brewery of Rochefort, Belgium.

Media and Growth Conditions

Yeasts were grown in YPS media (1% yeast extract, 5% peptone, and 10% sucrose) or in a 12°P industrial wort (12 g of extract per 100 g of wort).

Fermentations were carried out at 23 or 28°C, in agitated conical flasks or in 1-L tall EBC tubes, with a pitching rate of 10 millions cells per mL. The industrial brewery vessel (1 hL) was a rectangular stainless steel fermentor regulated at 23°C. The pitching rate was 3.3 millions cells per mL for each three strains

TABLE I
Comparison Between the Experimental Conditions of Fermentation Used to Study Amino Acid Uptake

	Jones and Pierce (9)	Palmqvist and Ayrapaa (14)	Ramos-Jeunehomme et al (15)	This Study
Strains	*Saccharomyces cerevisiae* Guinness 4200	*S. carlsbergensis* U15	*S. cerevisiae* 1278b and 2592, 59R	*S. cerevisiae* BRAS 291, BRAS 212 and BRAS 12 + industrial co-culture of 3 top fermentation yeasts
Stirring	Stirred (conical flask) and industrial vessel	Stirred and non stirred	Stirred	Stirred (conical flask), non stirred (EBC tube), and industrial vessel
Temperature	15.5 and 23°C	8-9°C	18°C	22 and 28°C
Medium	10-12°P	10°P	12°P	12°P
Group A	Glutamate, glutamine, aspartate, asparagine, serine, threonine, lysine, arginine	Asparagine, glutamine, serine, threonine	Glutamate, glutamine, aspartate, asparagine, serine, threonine, lysine, arginine, methionine	Aspartate, glutamate, serine, threonine, lysine, methionine (A'group)
Group B	Valine, methionine, leucine, isoleucine, histidine	Methionine, lysine, aspartate, leucine, glutamate, isoleucine, arginine	Valine, leucine, isoleucine, histidine	Valine, arginine, isoleucine, leucine, tyrosine, phenylalanine, tryptophane (B' group)
Group C	Glycine, phenylalanine, tyrosine, tryptophane, alanine	Valine, histidine, phenylalanine, alanine, tyrosine, tryptophane, glycine, proline	Glycine, phenylalanine, tyrosine, tryptophane, alanine	Glycine, alanine (C' group)
Group D	Proline		Proline	Proline

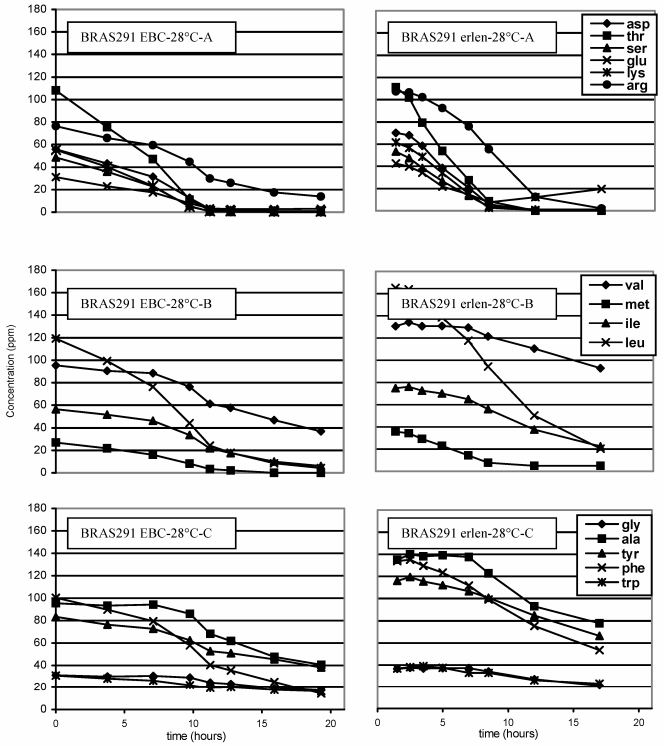

Fig. 1. Comparison of amino acid uptake by BRAS291 (lager yeast) between 1-L tall EBC tubes and 1-L conical flask fermentations conducted at 28°C. A, B, and C classes are as defined by Jones and Pierce (9). Because the experiments have been conducted on different batches of the same wort,, slight differences can be observed in the initial concentration of some amino acids. Asp = aspartate, thr = threonine, ser = serine, glu = glutamate, lys = lysine, arg = arginine, val = valine, met = methionine, ile = isoleucine, leu = leucine, gly = glycine, ala = alanine, tyr = tyronsine, phe = phenylalanine, and trp = tryptophane.

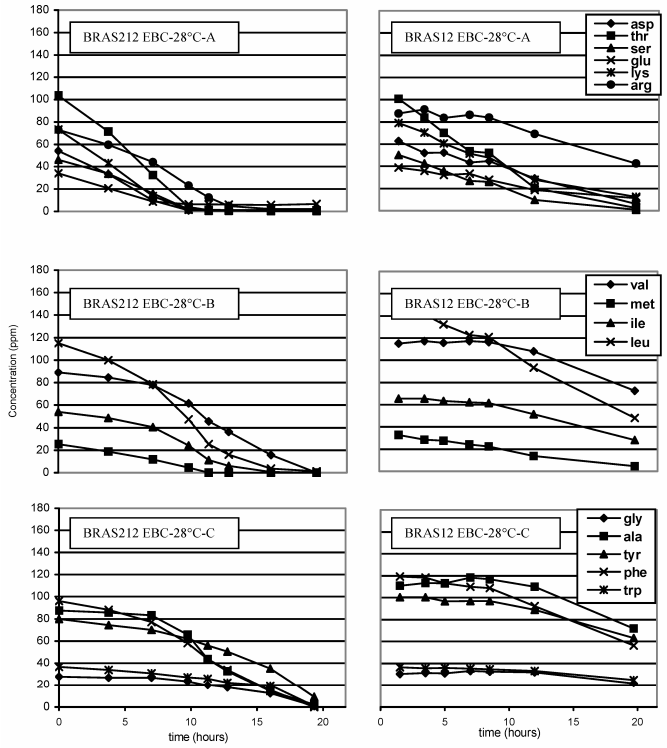

Fig. 2. Amino acid uptake by BRAS212 (ale yeast) and BRAS12 (lager yeast) in a 1-L tall EBC tube fermentation conducted at 28°C; A, B and C classes are as defined by Jones and Pierce (9). Because the experiments have been conducted on different batches of the same wort, slight differences can be observed in the initial concentration of some amino acids. Asp = aspartate, thr = threonine, ser = serine, glu = glutamate, lys = lysine, arg = arginine, val = valine, met = methionine, ile = isoleucine, leu = leucine, gly = glycine, ala = alanine, tyr = tyronsine, phe = phenylalanine, and trp = tryptophane.

of the co-culture. Each sample was immediately centrifuged to discard particles and frozen at –20°C.

Amino Acid Analysis

One milliliter of sample was centrifuged at 10,000 rpm (12,062 g) in a Sorvall SS-34 rotor for 15 min at room temperature. A 3-mL mixture including 1 mL of the supernatant, 540 µL of ultra-pure water (Milli-Q water purification system; Millipore, Bedford, MA), 140 µL of a 1% (w/v) TFA solution, 120 µL of 2.5 m*M* norleucine (internal standard), and 1,200 µL of methanol was centrifuged at 10,000 rpm for 5 min at room temperature in an Eppendorf centrifuge. The supernatant was flushed through a C18 Sep-Pack cartridge (Waters, Milford, MA) preactivated with 20 mL of methanol, 20 mL of 0.1% (w/v) TFA, 10 mL of an 80/20 0.1% (w/v) TFA/methanol solution, and 30 mL of air. The fraction eluting between 1.5 and 2.4 mL was collected.

Amino acids were separated by cation-exchange HPLC, post-derivatized with orthophtaldialdehyde in presence of mercaptoethanol and quantified by fluorescence as previously described by Dethier et al (3). The variation coefficient is in all cases under 5%.

RESULTS AND DISCUSSIONS

Saccharomyces cerevisiae strains BRAS 291, BRAS212, and BRAS12 were pitched into EBC tubes containing 12°P wort. The wort amino acid composition was monitored by HPLC throughout the fermentations that were conducted at 28°C. Table I compares the experimental conditions used in this work with those previously applied by Jones and Pierce (9), Palmqvist and Ayrapaa (12), and Ramos-Jeunehomme et al (15).

As depicted in Figures 1 and 2, some rules in agreement with those previously described by Jones and Pierce (9) emerge from our data. A 'critical time' (Tc), can be defined for each strain as the time necessary for complete consumption of a first group of amino acids. This group coincides with the A class described by M. Jones (aspartate, threonine, serine, glutamate, lysine, and arginine), plus methionine and minus arginine. After 10 hr, this group was indeed completely depleted, while 30% of the amino acids belonging to other classes still subsisted. According to Ramos-Jeunehomme et al (15), methionine should be effectively consumed after a few hours of fermentation. Tc also marked the beginning of consumption of a third group, which according to our results should only include glycine and alanine. Phenylalanine, tryptophane, and tyrosine, although classified by Pierce (14) in group C, in this study turned out to be partially used before the critical time, especially by BRAS291 and BRAS212. The other amino acids, classified in the intermediate group, seem to be slowly and gradually taken up without any lag phase. The new groups, thus defined and named A', B', and C' in this study, are depicted in Figure 3 for all three strains. Tc, close to 10 hr for BRAS291 and BRAS212, was much longer for BRAS12 (close to 20 hr).

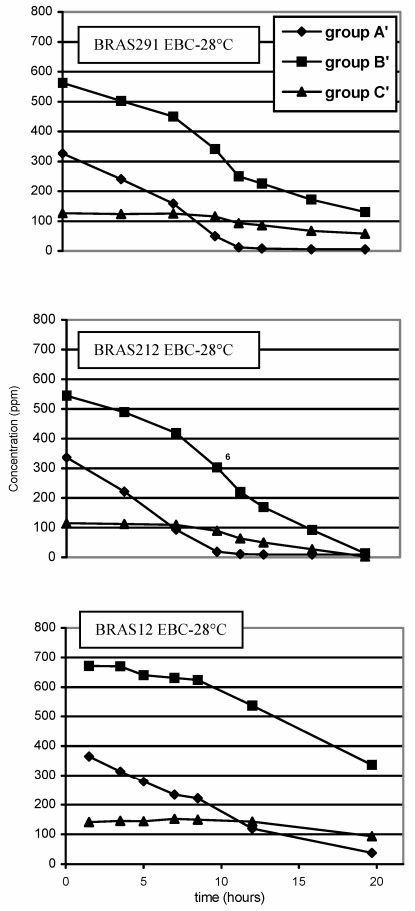

Fig. 3. Amino acid uptake by BRAS291 (lager yeast), BRAS212 (ale yeast), and BRAS12 (lager yeast) in a 1-L tall EBC tube fermentation conducted at 28°C. A', B', and C' classes are as defined in the current paper.

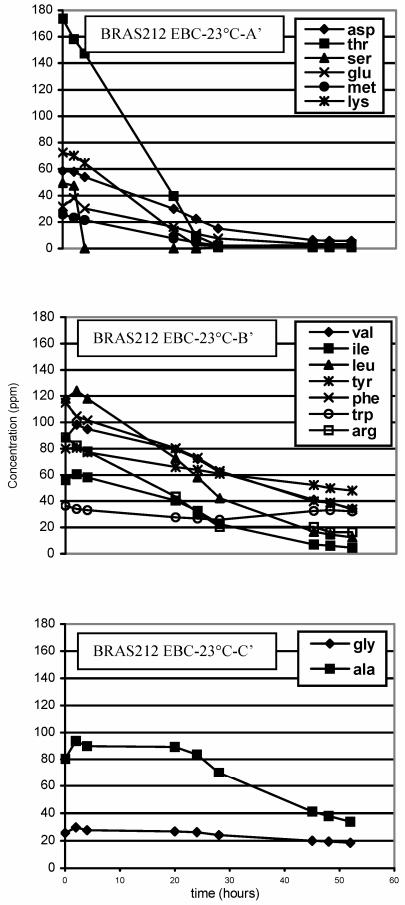

Fig. 4. Amino acid uptake by BRAS212 (ale yeast) in a 1-L tall EBC tube fermentation conducted at 23°C. A', B', and C' classes are as defined in the current paper. Asp = aspartate, thr = threonine, ser = serine, glu = glutamate, met = methionine, lys = lysine, val = valine = ile = isoleucine, leu = leucine, tyr = tyrosine, phe = phenylalanine, trp = tryptophane, arg = arginine, gly = glycine, and ala = alanine.

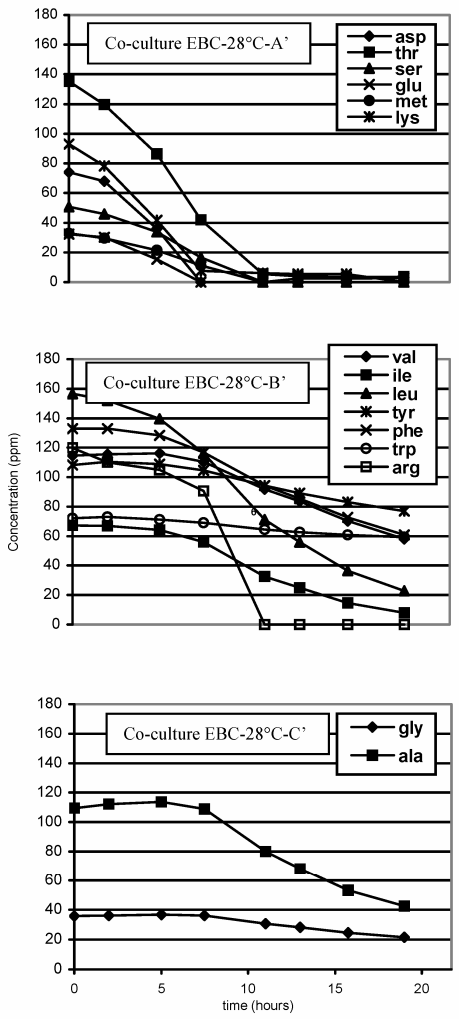

Fig. 5. Amino acid uptake by a co-culture of 3 ale yeasts in 1-L tall EBC tubes-fermentations conducted at 28°C; A', B', and C' classes as defined in the current paper. Asp = aspartate, thr = threonine, ser = serine, glu = glutamate, met = methionine, lys = lysine, val = valine = ile = isoleucine, leu = leucine, tyr = tyrosine, phe = phenylalanine, trp = tryptophane, arg = arginine, gly = glycine, and ala = alanine.

As for previous works done in this area, concentrations in the wort are the combination of the absorption and excretion mechanisms, especially in the case of aspartate and glutamate. Nevertheless, we can assume that excretion is quite negligible compared to the 2,100 ppm of amino acids found in the wort.

BRAS291 fermentations carried out in conical flasks gave results similar to those obtained with EBC tubes (Fig. 1). Of course, stirring increased the uptake kinetics, leading to a lower Tc value (8 hr instead of the 10 hr observed in EBC tubes).

To understand why our results differed slightly from those published by Pierce (14), we also fermented BRAS212 at 23°C instead of 28°C. As shown in Figure 4, the temperature did not significantly influence the uptake sequence.

Finally, three yeast strains currently used in co-culture at a Belgian brewery were investigated in 1-L tall EBC tubes and industrial vessels. Figure 5 clearly shows that the pattern obtained with the co-culture was similar to those previously obtained with the BRAS291, BRAS212, and BRAS12 strains. Once more, a Tc close to 10 hr could be defined.

Industrial fermentations were carried out in a rectangular vessel at 23°C. These experiments confirmed major divergences between groups A' and C'. As shown in Table II, 71 to 99% of the A' group was consumed in the first 15 hr, while as much as 50% of the C' group was still present at the end of this period. However, the A' group amino acids revealed were not completely consumed in the industrial vessel after 15 hr at 23°C, contrarily to what happened in the EBC-tube fermentations conducted at 28°C. The design of the fermentor vessel is probably responsible for the extra time of 3 hr needed to complete total depletion of the A' group. Surprisingly, arginine was consumed by the co-culture as a A' group amino acid in both EBC tube and industrial vessel. The uptake of this alkaline amino acid was previously depicted as very complex by Jones and Pierce (9), its evolution depending on flocculation abilities of the strains used.

In this study, the proposed critical time parameter should help brewers to better monitor their fermentation. However, molecular biology experiments are still needed to better understand the role of the sensors and permeases leading to this sequential assimilation of amino acid as seen in wort fermentation. Huge differences remain between molecular biology data on permeases and the model of sequential assimilation of amino acids. Even if it is now clear that GAP1p is not expressed in the brewery conditions (12) and that Ssy1p has been revealed as a key sensor during

TABLE II
Amino Acid Concentration (ppm) in Fermented Wort (1-L Tall EBC Tube at 28°C or 1-hL Rectangular Vessel at 23°C)

	EBC Tube			Industrial Vessel		
Group	**0 hr**	**15.75 hr**	**Difference (%)**	**0 hr**	**15 hr**	**Difference (%)**
A'						
Aspartate	74.0	0.0	100.0	143.4	23.3	83.8
Threonine	135.4	3.4	97.5	331.9	74.4	77.6
Serine	50.8	0.0	100.0	136.7	30.9	77.4
Glutamate	32.4	2.4	92.6	132.1	7.5	94.4
Lysine	92.9	5.4	94.1	193.6	1.2	99.4
Methionine	32.9	0.0	100.0	76.1	21.9	71.3
B'						
Valine	114.7	70.1	38.9	266.1	126.0	52.7
Arginine	119.9	0.0	100.0	284.3	89.9	68.4
Isoleucine	67.3	14.5	78.4	160.4	66.6	58.5
Leucine	156.5	36.6	76.6	372.7	137.0	63.2
Tyrosine	108.2	82.9	23.3	231.5	111.1	52.0
Phenylalanine	132.8	72.8	45.1	290.4	130.9	54.9
Tryptophane	72.2	60.9	15.6	90.5	45.0	50.3
C'						
Glycine	35.8	24.5	31.4	72.8	36.0	50.6
Alanine	109.5	53.3	51.4	230.6	114.8	50.2

fermentation (13), the role of each Ssy1p-dependent permease remains to be described.

CONCLUSIONS

Tc, defined as the time by which the amino acids of group A' are totally consumed, emerged from all our experiments. Group A' coincides with the A class described previously by Jones and Pierce (9) (aspartate, threonine, serine, glutamate, lysine, and arginine), plus methionine and minus arginine. Tc also corresponds with the beginning of consumption of a group (called C' in the current work) that includes only glycine and alanine. All other amino acids, defining the B' group, are slowly and gradually taken up without any lag phase.

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