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Improvement in RNA Extraction from *S. cerevisie* by Optimization in the Autolysis and NH_3 Hydrolysis

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ABSTRACT

The optimization of autolysis of Saccharomyces cerevisiae *from brewery was studied aiming at the maximum ribonucleic acid extraction and yeast extract production. The best conditions for yeast autolysis was 55.2ºC, pH= 5.1 and 9.8% NaCl for 24h of processing, without the NH_3 use. In these conditions, the RNA yield was 89.7%, resulting in 51.3% of dehydrated yeast extract with 57.9% protein. The use of 12.2% NH_3 at 60ºC after autolysis (8h) and plasmolysis (8h) was not viable due to the reduction in the RNA yield from 89.7to78.4%. On the other hand, the thermal shock at 60ºC for 15 minutes prior to autolysis provided an increase in the yield from 89.7 to 91.4%. The autolysis, including NaCl plasmolysis in the optimized conditions was efficient, economic and with short time, thus usable for industrial purpose to obtain more valuable products such as yeast extract enriched in RNA and/or protein, for different applications.*

Key words: Autolysis, NaCl plasmolysis, yeast extract, RNA extraction, nucleotides

INTRODUCTION

The yeast extract is a natural additive widely used as nutritious and flavor complement for food formulation, as dehydrated soaps, sauces, biscuits and seasonings (Oliveira, 2001). It is rich in protein and vitamins and also contains fibers, fatty acids, minerals and nucleotides (Vilela et al., 2000). Yeast extract could be used as protein sources in human nutrition, but the presence of RNA in relatively high concentration limits its use. The daily ingestion of RNA in adult could not exceed 2 g, due to nucleic acids when hydrolyzed produce uric acid which causes the gout (Duk-Hee et al., 2002). On the other hand, nucleotides improve the immune system in children and animals. Nowadays, the use of antibiotics in feed is being replaced by nucleotides and mannanoligosacharide from yeast cell wall (Rossi et al., 2007).

Yeast extract is obtained from a pure culture of yeasts such as *Saccharomyces cerevisiae*, or recovered biomass of fermentative process from cell fragmentation, followed by concentration and drying of soluble fraction (Sgarbieri et al., 1999). The insoluble cell wall is rich in glucan and mannan, and when purified has great application as thickening and substitute of fatties in dietetic food (Chaud and Sgarbieri, 2006).

The yeast biomass recovered from beer production is an interesting residue, since it is abundant and low cost. It contains 45-65% proteins and 8-12% nucleic acids (Halász and Lastity, 1991). The RNA

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when extracted in a polymeric form can be purified and utilized for the production of flavor enchancings, GMP and IMP (Sombutyanuchit et al, 2001).

The use of rich extracts in 5'-ribonucleotides, GMP and IMP, has increased intensely for animal and human nutrition. This is due to the convenience for use in processed foods, since there is an increase in consumers demand for more variety of better flavor products, development of food processed and search for diets, including nucleotides and products without glutamate (Yamauchi, 2002). Industrially, the autolysis is the main method for the production of yeast extract and cell wall (Jimenez et al., 1993). According to Tnanekawa et al., (1981), the acidic process is more favorable for a major preservation of RNA in its polymeric form, besides minimizing the loss of proteins and amino acids. The parameters of autolysis were studies by Sugimoto et al., (1973) and Jimenez et al., (1993). However, the time of autolysis is not short and new technologies with enzymes are being developed to improve the yeast extract production (Zhang et al., 2008). Methods utilizing alkalis for the production of concentrate rich in protein and low content of RNA were studied by Andreu et al., (1987) and Behalová et al., (1991). The authors proved the efficiency of ammonium compounds in the reduction of RNA on biomass, however, with the loss in protein content. In Brazil, the ethanol distilleries and Breweries are exporting the yeast biomass as flour for feed with low price. The improvement of the technology of fractionation and purification of this product in others most valuable is strategic. This is in accordance to the concept of biorefinery, i.e., co-production of transportation biofuels, bioenergy and marketable chemicals from the renewable biomass (Cherubini and Ulgiati, 2010). In this work, the yeast technology was studied by the optimization of the parameters of autolysis, plasmolysis and alkaline hydrolysis, aiming to improve the RNA extraction of brewery's yeast and yeast extract production. Also the RNA.recovery from autolysate yeast biomass was evaluated fractioned by precipitation with pH (isoeletric point) and ethanol.

MATERIALS AND METHODS

Microorganism

Saccharomyces cerevisiae obtained from the Malta brewery (Assis-SP) was used in this work with the cell viability of 97%. A cell suspension constituted by 15% (w/v) of yeast was washed and debittered. The yeast washing was carried out with distilled water in two operations of vaccum filtration process in a 15μm nylon filter. Subsequently, the biomass was centrifuged at 2500 x g for 15 min and re-suspended in 0.2% NaOH solution in a ratio of 1:1 (v/v). After that, the yeast suspension was homogenized for 30 min, centrifuged again and re-suspended with distilled water to obtain neutral pH of yeast suspension.

Reagents

Ribonucleic acid from baker's yeast-*S.*cerevisiae tipe III, GMP, IMP and Orcine were obtained from Sigma-Aldrich. NaOH, NH_4OH, NaCl, H_3PO_4, $FeCl_3.6H_2O$, HCl and red of eritrosine were obtained from Merck.

Equipaments

The following equipments were used in this work: HPLC (Waters 27475 - multi λ fluorescence detector), refrigerated centrifuge (Hitachi - Japan), optical microscope (Olympus – São Paulo- Brazil), Neubauer camara (Laboroptik), mechanical agitator (Fisatom – São Paulo - Brazil), UV visible spectrophotometer (Fento – Piracicaba - Brazil), nylon synthetical tissue opening 15, 20, 45 and 130μ (Sefar Tenyl), analytical balance (Gehaka), drying stove, thermostatized bath, pHmeter, vacuum filtration system and evaporator (Tecnal Piracicaba – Brazil).

Analytical methods

The analytical procedures followed were: Ribonucleic acid (Herbert et al., 1971), total nitrogen/crude protein, dry matter, ashes and fiber (AOAC, 2000), and Cell viability (Bonneu et al., 1991). The RNA extraction yield was calculatedas g RNA on autolysed biomass (without cell wall) per g RNA (non autolysed biomass) x 100 and expressed in percentage.

Yeast autolysis

The yeast autolysis and subsequent NH_3 hydrolysis were studied by factorial design according to Box and Benken (1989). They were evaluated by surface methodology utilizing the Software Statistic 5.1. A cell suspension constituted by 15% (w/v) of yeast was submitted to different treatments for cell rupture. The selection of the best results from the previous assays was used for next one. The proc-

ess optimization of autolysis, plasmolysis and NH_3 hydrolysis were finished by four assays in triplicate, according to the experimental design of Table 1. The conditions were: I) autolysis (4h) process at 40 to 60°C and pH 4.0-6.0, II) autolysis (8h) and plasmolysis (8h) process at 40 to 60°C and 6-8% (w/v) NaCl, III) autolysis (8h) and plasmolysis (8h) at 50 to 60°C and 8-12% (w/v) NaCl followed by 7-11% (w/v) NH_3 hydrolysis (15 min). IV) the autolysis (12h) and plasmolysis (12h) at 45-65°C and 6-14% (w/v) NaCl, followed by 8-16% NH_3 hydrolysis at 60°C (15 min). After the optimizations of autolysis, plasmolysis and alkaline hydrolysis were carried out as follows: 1) yeast autolysis (12h) and plasmolysis (12h) at 55.2°C, pH 5.1 and 10.3% (w/v) NaCl, 2) thermical shock of biomass at 68°C for 5 min followed by autolysis at the same conditions of the first treatment, 3) only 12.5% (w/v) NH_3 hydrolysis at 60°C for 15 min.

Recovery of cell fractions and RNA

After autolysis, plasmolysis and NH_3 hydrolysis, the autolysate yeast suspension was centrifuged at 5,000 x g for 20 min and the samples of dehydrated fractions (extract and cell wall) were evaluated for the chemical composition, biomass yield and RNA extraction yield. RNA was recovered from the yeast autolysate that was adjusted to pH 2.2 with 20% (w/v) phosphoric acid, by adding two volumes of ethanol. After precipitation, the suspension was centrifuged (5000 x g/30 min), the supernatant (without RNA) was discarded and RNA pellet was dried.

Table 1 - Independent variables levels of yeast autolysis (temperature and pH) and plasmolysis (NaCl)

Assays	Independent Variables	Dependent Variable*	Levels					Factioned Factorial
			(-2)	(-1)	(0)	(+1)	(+2)	
I	T(°C)	Y%		40	50	60		$3^{**(2-0)}$
	pH			4	5	6		
II	T(°C)			40	50	60		$3^{**(2-0)}$
	NaCl (% w/w)			0	4	8		
	T(°C)			50	55	60		
III	NaCl (% w/w)	Y%		8	10	12		$3^{**(3-0)}$
	NH_3 (% w/w)			7	9	11		
	T(°C)		45	50	55	60	65	
IV	NaCl (% w/w)	Y%	6	8	10	12	14	$2^{**(5-2)}$
	NH_3 (% w/w)		8	10	12	14	16	

* Dependent variable: Y% (g RNA yeast extract / g RNA yeast biomass x 100).

RESULTS AND DISCUSSION

Table 2 shows the *p* values indicating the significance of the process to $p<0.05$ to the pH and temperature effects in RNA extraction yield of assay I. The best pH was 5.1 and temperature between 51.5 and 56.0°C (Fig. 1). These results were obtained in 4h of autolysis without the NaCl for plasmolysis which were similar to that previously reported. Sugimoto et al., (1973) mentioned that a good autolysis process will be able at pH 4.0. Béhalová et al., (1991) found pH 3.0-5.0 as best range for a high autolytic activity. In yeast autolysis at 30°C for 48 h, there was a decrease in nitrogen and nucleotides concentration and higher residual proteolytic activity, preserving the autolytical ability.

Table 2 - Effect of pH and temperature on RNA extraction yield (Assay I).

Aplied levels	Results(1)		
	The best band	The best point	*p*
T (°C)	51.5-56.0	53.5°C	0.001798*
pH	4.6-5.6	5.1	0.047707*
Interaction effect (T°C x pH)			0.000009*

(1): 4h of autolysis without NaCl and NH_3 (*) $p<0.05$ (significative).

Table 3 shows the *p* values indicating the significance of the process to the pH and temperature effects on RNA extraction yield of assay II. The linear and quadratic effects for the temperature and concentration of sodium chloride, as well the T *versus* NaCl interaction were significant with $p<0.05$ for RNA yield. There was a synergism between the studied variables. However, further studies were needed, since NaCl used in the plasmolysis was not enough.

Table 3 - Effect of temperature and NaCl on extraction yield of RNA by the processes of autolysis/plasmolysis (Assay II).

Applied levels	Results(1)		
	The best band	The best point	*p*
T (ºC)	49.0-61.0	55.0ºC	(L) 0.000033* (Q) 0.000208*
NaCl (% w/w)	≥ 8.0	> 8.0	(L) 0.000088* (Q) 0.001124*
Interaction effect (TºC x NaCl)			(L) 0.036470*

(1): Time of processes: 4h autolysis + 4h autolysis/plasmolysis (*) $p<0.05$ (significative).

Figure 1 indicated the pH obtained in assay I and the best range was between 4.6 to 5.6, with the optimum value of 5.1, associated to a temperature range varying from 51.5 to 56.0ºC, with the optimum at 53.5ºC. Figure 2 showed that the NaCl quantity expressed in dried yeast matter tended to an up value of 8% (w/w dried yeast). The best temperature range was between 49 to 61ºC, with optimum at 55ºC, showing the necessity of additional studies (assay III).

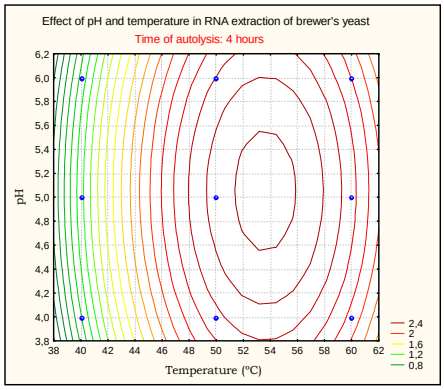

Figure1 - Effect of pH and temperature in RNA extraction of the biomass (Assay I).

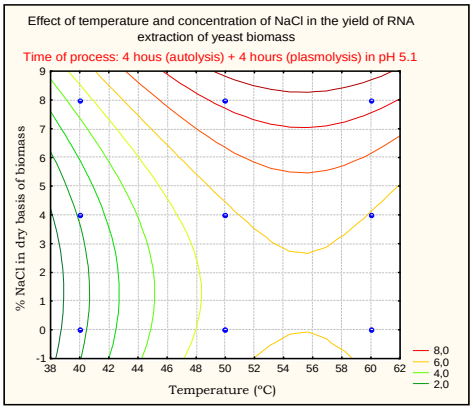

Figure 2 - Effect of temperature and NaCl in the yield of RNA extraction (Assay II).

Tables 4 and 5 show the *p* values indicating the significance of the process to the pH and temperature effects, respectively on RNA extraction yield in assay III.

The linear effects were significant for temperature, NaCl, NH_3 and NaCl x NH_3 interaction. The best ranges were temperature 52.5 to 58.5ºC and optimum as 55.5ºC, NaCl 8.7 to 11.8% w/w dried yeast with optimum as 10.5%, NH_3 (minimum 9.2% w/w dried yeast) and 71.8 to 76.7% for RNA extraction (Table 5).

Table 4 - RNA extraction yield of yeast biomass (Assay III).

Experiments	T (ºC)	NaCl (% w/w)	NH_3 (% w/w)	(Y % w/w)
1	50	8.00	7.00	57.40
02	50	8.00	9.00	60.90
03	50	8.00	11.00	63.21
04	50	10.00	7.00	55.20
05	50	10.00	9.00	68.87
06	50	10.00	11.00	71.43
07	50	12.00	7.00	53.11
08	50	12.00	9.00	64.12
09	50	12.00	11.00	67.53
10	55	8.00	7.00	59.86
11	55	8.00	9.00	72.52
12	55	8.00	11.00	65.47
13	55	10.00	7.00	61.54
14	55	10.00	9.00	67.38
15	55	10.00	11.00	83.79
16	55	12.00	7.00	59.45
17	55	12.00	9.00	67.82
18	55	12.00	11.00	77.90
19	60	8.00	7.00	61.67
20	60	8.00	9.00	68.35
21	60	8.00	11.00	59.46
22	60	10.00	7.00	58.40
23	60	10.00	9.00	63.67
24	60	10.00	11.00	74.56
25	60	12.00	7.00	57.93
26	60	12.00	9.00	63.57
27	60	1.00	11.00	73.68

Y (%): Extraction yield (g RNA yeast extract/g RNA yeast biomass x 100).

Table 5 - Effect of temperature, NaCl and NH_3 on RNA extraction (Assay III).

Applied levels	Results(1)		
	The best band	The best point	*p*
T (ºC)	52.5-58.5	55.50	0.015034*
NaCl (w/w dried biomass)	8.7-11.8	10.5	0.012429*
% NH_3 (w/w dried biomass)	>9.2	>9.2	0.000012*
TºC x % NaCl			0.858566
TºC x % NH_3			0.624033
NaCl x NH_3			0.009747*

(1) Time of processes: autolysis (8 h), plasmolysis (8 h) and NH_3 (15 min) (*) $p<0.05$ (significative)

Figures 3 to 5 show the interactions among the optimized variables and the respective RNA extraction yields obtained experimentally. The best determined point of the autolysis were 55.5ºC, plasmolysis 10.5% (w/w) NaCl and minimum of 9.2% (w/w) NH_3 hydrolyis in yeast extract.

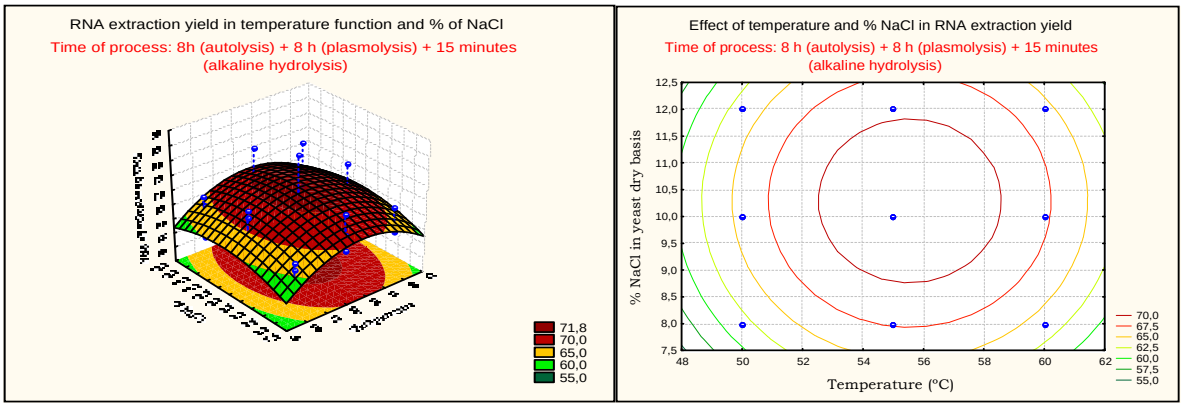

Figure 3 - Interaction between temperature of autolysis and NaCl of plasmolysis in RNA extraction yields (Assay III).

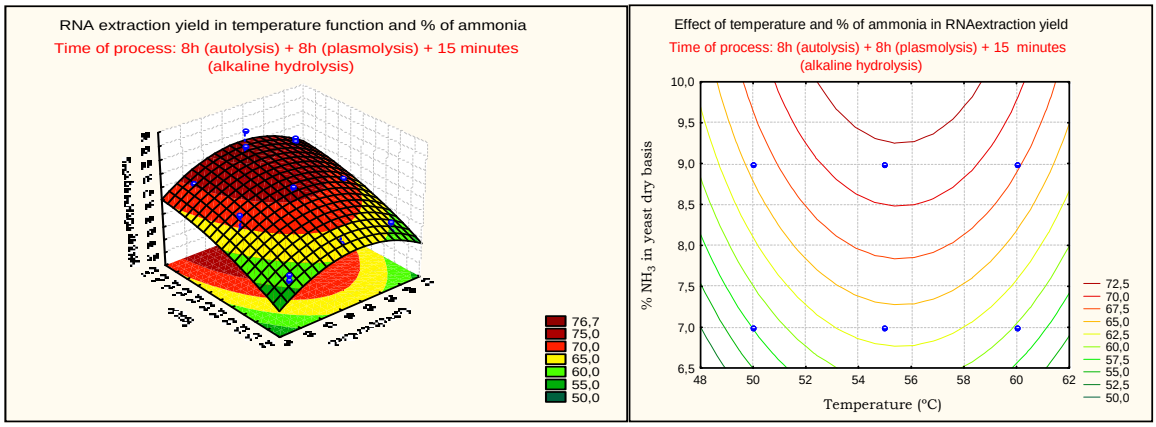

Figure 4 - Interaction between temperature of autolysis and NH_3 hydrolysis in RNA extraction yield (Assay III).

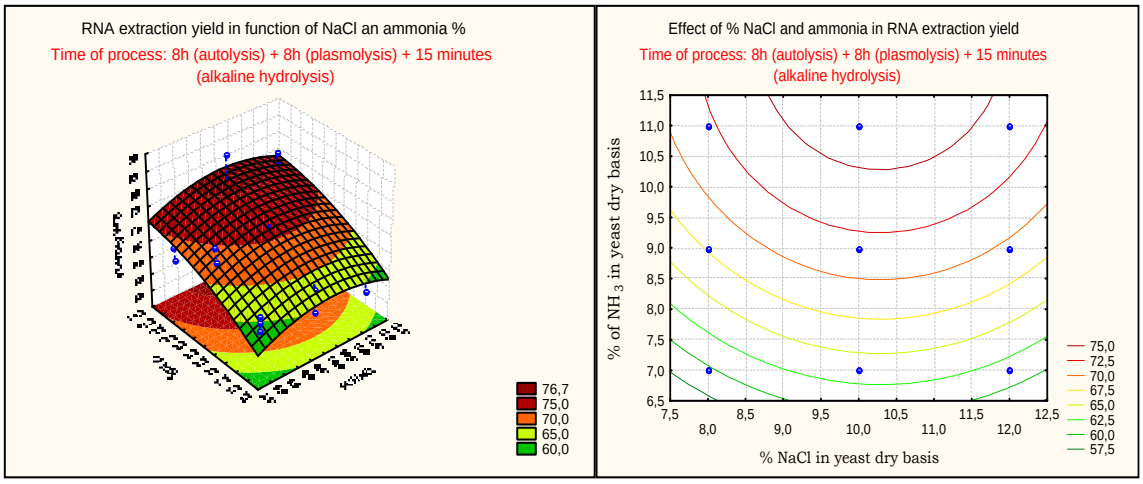

Figure 5 - Interaction among NaCl of plasmolysis and NH_3 hydrolysis in RNA extraction yield (Assay III).

Figures 6 to 8 show the interactions among the optimized variables and the respective RNA extraction yields obtained experimentally (Assay IV). In the Table 7, the *p* values indicated the significance of the process to the T, NaCl and NH_3 in RNA yield in assay IV. All the variables presented significant effects with $p<0.05$, regression deviation was not significant with lack of fit and a determination coefficient R^2 of 0.939, indicating that the generated mathematical model from the experimental data was appropriate. The optimized values in assay IV were temperature range from 53.0 to 57.5°C with the optimum as 55.1°C, 8.7 to 11.2% (w/w) of NaCl (the best in 9.8%) and 11.0 to 13.5% (w/w) of NH_3 with the optimum as 12.2%, giving the maximal RNA extraction yield of 81.92% (w/w) determined by the model and 84.93% observed experimentally (Table 6).

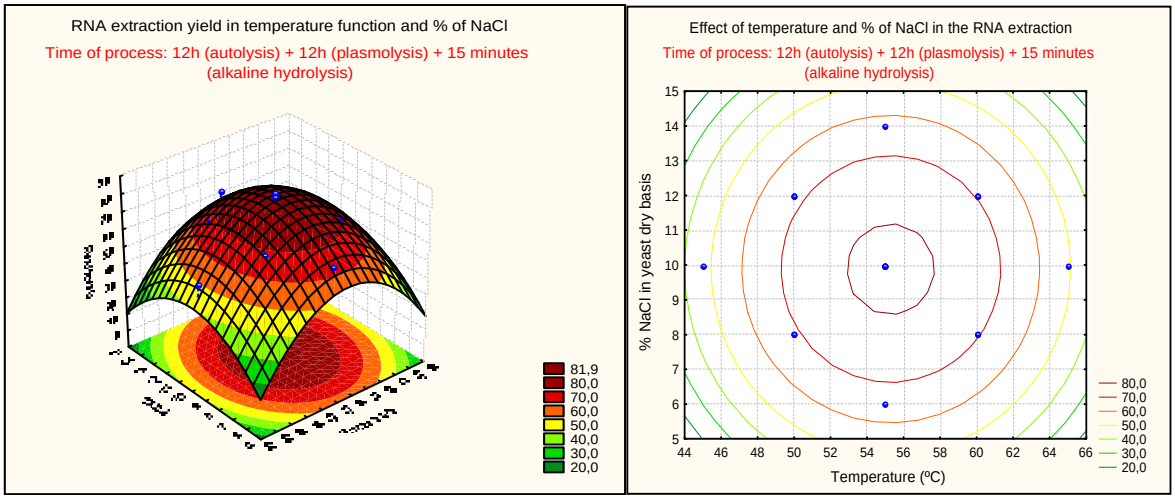

Figure 6 - Interaction among temperature of autolysis and NaCl of plasmolysis on RNA extraction yield (Assay IV).

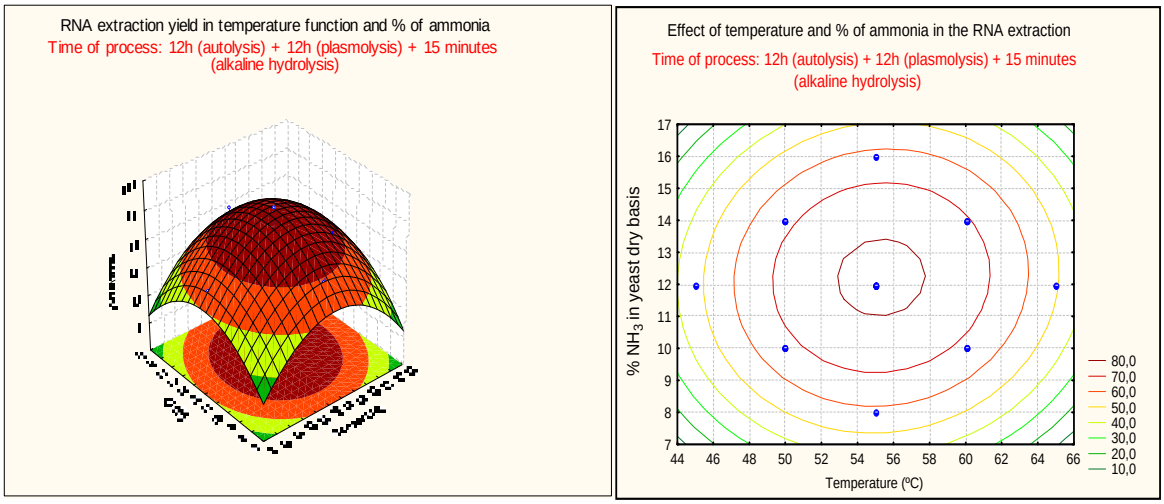

Figure 7 - Interaction among temperature of autolysis and NH_3 hydrolysis on RNA extraction yield (Assay IV).

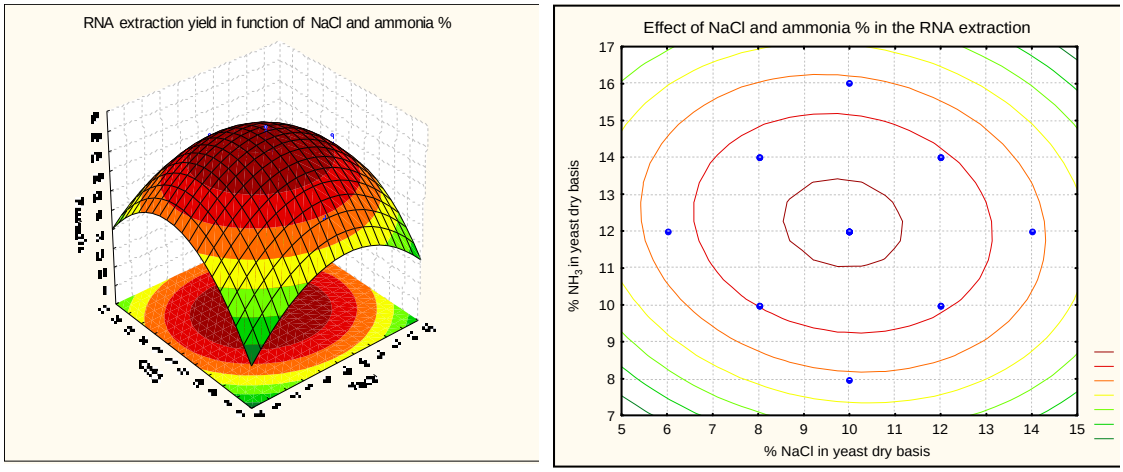

Figure 8 - Interaction among NaCl of plasmolysis and NH_3 hydrolysis in RNA extraction yield (Assay IV).

Table 6 - RNA extraction yield in different combinations of temperature, NaCl and NH_3 (Assay IV).

Experiments	T (ºC)	% NaCl	% NH_3	(Y%)
01	50.00	8.00	10.00	61.54
02	50.00	12.00	14.00	53.19
03	60.00	8.00	14.00	66.30
04	60.00	12.00	10.00	63.16
05	55.00	10.00	12.00	79.67
06	50.00	8.00	14.00	68.26
07	50.00	12.00	10.00	58.71
08	60.00	8.00	10.00	65.27
09	60.00	12.00	14.00	68.31
10	55.00	10.00	12.00	84.93
11	45.00	10.00	12.00	51.16
12	65.00	10.00	12.00	48.31
13	55.00	6.00	12.00	62.19
14	55.00	14.00	12.00	67.11
15	55.00	10.00	8.00	58.35
16	55.00	10.00	16.00	63.44
17	55.00	10.00	12.00	82.23

Y (%): RNA extraction yield = g RNA in yeast extract/ g total RNA in yeast biomass.

Table 7 - Effect of temperature, NaCl and NH_3 on RNA extraction yield (Assay IV).

Applied levels	Results(1)		
	The best band	The best point	*p*
T (ºC)	53.0-57.5	55.10	0.0055260*
NaCl (w/w dried biomass)	8.7-11.2	9.80	0.0173060*
NH_3 (w/w dried biomass)	11.0-13.5	12.20	0.0119350*
Lack of fit			0.2496250

(1) 12h (autolysis) + 12h (plasmolysis) + 15 min (hydrolysis) (*) $p<0.05$.

Table 8 and Figures 9 and 10 show the RNA extraction yields by different processes under the optimized conditions. The data indicated that in 24h of autolysis with 9.8% NaCl, the RNA extraction yield was 89.7%. The 12.2% ammonia addition after 24h for 15 min more at 60ºC increased it by only 3.9%. This indicated that the use of ammonia was not necessary. It should also be noted that NH_3 causes a strong browning of the extract.

Table 8 - RNA extraction yield by the yeast autolysis and alkaline hydrolysis process in optimized conditions.

Process Yield	Process time					
	8h	10h	12h	14h	24h	16h
RNA Yield - autolysis	21.16	34.75	47.84	65.41	80.27	89.72
RNA Yield - NH_3 Hydrolysis(*)	57.24	42.65	36.88	22.58	13.84	3.88
Global yield	78.40	77.40	84.72	87.99	94.11	93.60

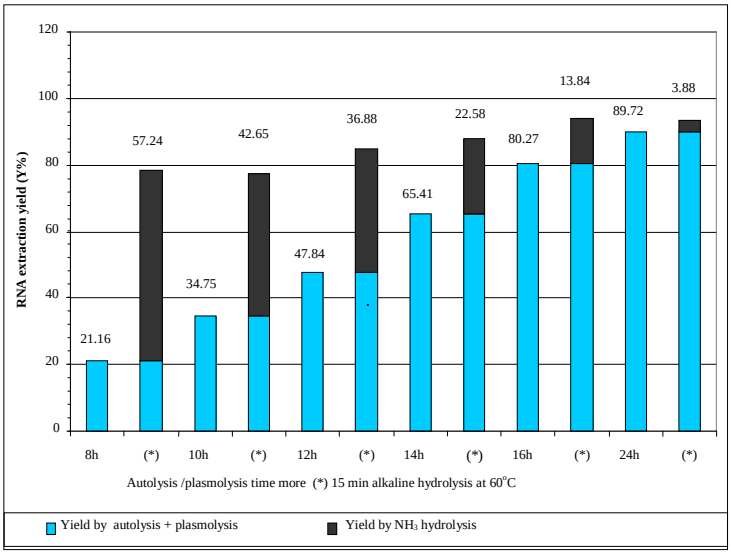

Figure 9 - Time evaluation and RNA extraction yield on combined processes.

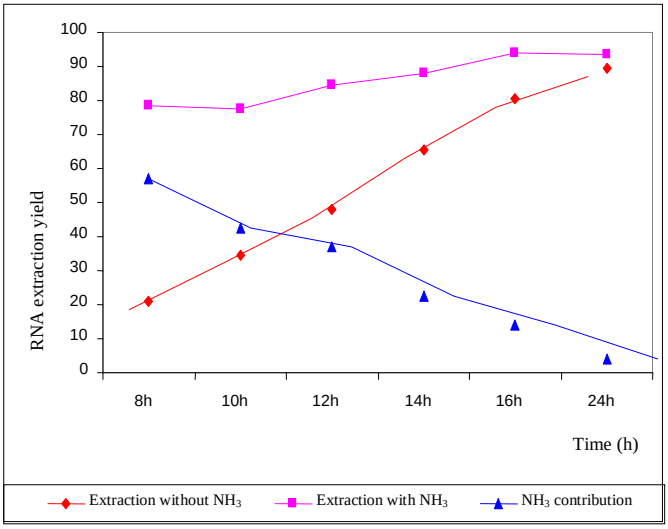

Figure10 - RNA extraction yield by different processes on Saccharomyces cerevisiae from brewer's biomass.

Table 9 shows the chemical composition and mass balance of biomass autolysis, dried yeast extract and cell wall fractions. The dried yeast extract yield based on total biomass was 51.3% (w/w dried yeast) with protein content of 57.9% (w/w). The dried cell wall yield was 48.7% (w/w dried yeast) with 21.7% (w/w) of protein. There was a loss of 2.9% protein.

Table 9 - The chemical composition and mass balance of Brewer's yeast biomass, dried yeast extract and cell wall from autolysis/plasmolysis process.

Chemical compostion	Biomass totality	Dried extract	Cell wall
Mass (g)	100.00	51.30	48.70
Protein (% w/w)	43.24	57.95	21.76
RNA (% w/w)	9.70	14.17	2.83
Fiber (% w/w)	28.90	2.40	nd
Ash	7.90	17.13	nd
Nd	10.26	8.35	75.41

nd: non determined.

Table 10 shows the RNA recuperation balance by the fractioned precipitation at pH 4.3 (protein) and 2.0 with two volumes of ethanol. The RNA recovery was evaluated from the extracts by the following processes. The RNA yield was 78.8% to 91.4%. Otherwise, there was a considerable loss of polymeric RNA, mainly when ammonia was utilized. The concentrations of GMP + IMP determined in dried extract base were 0.39, 1.5 and 0.3%, respectively for the methods 1^{st}, 2^{nd} and 3^{rd}.

Table 10 - RNA extraction and recovery from autolysate yeast biomass by fractioned precipitation in pH (isoeletric point) and ethanol.

Method (*)	RNA extraction of biomass (%)	IMP+GMP (%) Yeast extract	RNA recovery (%)	RNA loss in precipitation (%)
1	87.45	0.39	15.47	25.44
2	91.40	1.50	13.80	24.48
3	78.80	0.30	7.42	29.76

(*) Extraction method: : (1) autolysis with 9.8% NaCl for 24 h, (2) thermical chock at 68ºC for 5 minutes followed by autolysis for 24 hs, (3) alkaline hydrolysis with 12.2% NH_3 at 60ºC for 15 minutes.

DISCUSSION

The optimization of the parameters involved in the autolysis, plasmolysis and alkaline hydrolysis of the *S. cerevisiae* biomass from the brewery was studied. It was possible to determine with more precision, and with correlations between parameters, the best conditions to achieve the maximum yield of nucleic acids from the yeast extract, particularly RNA from the yeast biomass. The best conditions (Tables 2, 3) were temperature of 55ºC and pH 5.1 to obtain the extracts rich in RNA. According to Oliveira et al., (1997) and Oliveira and Oliva-Neto (1999), the best conditions to obtain autolysis was 45-55ºC. This was due to the different enzymes acting at different best temperatures for this process. In this process the total time for autolysis and plasmolysis was short (up to 24 h) to obtain the maximum release of soluble RNA. This was much smaller than those (3-7 days) obtained by Sugimoto et al. (1973) and Oliveira and Oliva-Neto, 1999. The plasmolysis with 9.1% NaCl, and subsequent autolysis held in test IV, had a great influence to obtain a yield of 51% extract with high protein content (58%). In these conditions, just 16 h of processing (12 h plasmolysis and 4 h of autolysis) was needed to obtain 80% of RNA yield (Fs 9 and 10). Enzymatic hydrolysis with 2.5% papain and 0.025% lyticase, which was more expensive process, produced yeast extracts with high solid recovery of 61.95% and but only 98.39 g/l protein. Plasmolysis by the salts or solvents produced the cells which were morphologically unchanged, but with free passage for low weight molecules in the plasma membrane, which stimulated the autolysis of cell wall (Fenton, 1982). The use of 9.1 % NaCl and other optimized conditions in the present work decreased the autolysis time, improved RNA extraction and showed no need to use the alkaline

hydrolysis. Moreover, ammonia caused undesirable browning reactions in the yeast extract, which was required at 11.0 to 13.5% (w/w) concentration (table 7) making this process expensive. Other authors (Jimenez et al, 1993, Sugimoto et al 1973) found 15-30% NaCl for yeast autolysis, better values in relation of the present work, but this difference probably was due to the other optimized parameters. The heat shock prior to these treatments was also favorable to increase the RNA extraction, since it achieved the maximum extraction of 91.4% (Table 10). Aiming to scale up from these presented parameters, it would be necessary to test the RNA extraction on a pilot scale, including other studies such as precipitation by pH in isoeletric point, and ethanol to produce the yeast extract enriched in protein and free from RNA for nutritional purpose and RNA extracts for flavor or immunostimulant purpose.

CONCLUSIONS

1. By the optimization of the parameters of autolysis and NaCl plasmolysis of *S. cerevisiae* from the brewery biomass, it was possible to reduce the time of the process and target the autolysis to obtain the maximum RNA extraction.
2. The NH_3 hydrolysis was efficient in RNA extraction but less than the optimized autolysis. The combination of these processes was not necessary when optimized autolysis was processed in 24 h time, as almost 90% of RNA was extracted from the biomass.
3. The heat shock prior to autolysis also was favorable to increase the RNA extraction, since it achieved the maximum extraction of 91.4%.
4. The autolysis, including NaCl plasmolysis in the optimized conditions was efficient, economic and relatively rapid, thus, usable for industrial purpose to obtain the yeast extract enriched in the RNA and protein. Further studies on the separation could produce the extracts enriched in RNA without protein or vice verse for different commercial applications.

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