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Optimization of the RNA Content Reduction *in Saccharomyces cerevisiae*

Optimiranje smanjenja udjela RNA u kvascu *Saccharomyces cerevisiae*

Sunčica Beluhan and V. Marić

Faculty of Food Technology and Biotechnology,
University of Zagreb, Croatia

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Summary

Yeast as a source of protein for human consumption is limited by its relatively high nucleic acid content.

In this work we have studied the problem of RNA extraction in yeast ***Saccharomyces cerevisiae*** *(fresh baker's yeast) by optimization of the concentration of alkali solution (NH_4OH). Extractions with 5, 10 and 15 % of NH_4OH (referring to yeast dry matter) were performed for 15 minutes at 45–80 °C (step 5 °C) measuring losses of protein and biomass and percent of nucleic acid reduction. With relatively low mass ratio (10 % NH_4OH) at 60 °C, a final RNA content of 1.73 % was obtained after an extremely reduced extraction time of only 15 minutes.*

After the extraction procedure, RNA was recovered by fractional precipitation lowering pH (1^{st} step pH = 4.9; 2^{nd} step pH = 2.0) by addition of HCl. Two solid fractions were obtained and RNA content in the precipitates was analyzed. The yield of RNA (g RNA obtained per g RNA in native yeast) in the second precipitate was 72.1 %. Purity of the second precipitate was 69.7 %.

Sažetak

Uporaba kvasca, kao izvora bjelančevina za ljudsku prehranu, ograničena je velikom koncentracijom nukleinskih kiselina.

U ovom radu je proučavan problem ekstrakcije RNA iz biomase kvasca ***Saccharomyces cerevisiae*** *(svježeg pekarskog kvasca) optimiranjem koncentracije lužine (NH_4OH). Provedeni su postupci s 5, 10 i 15 % NH_4OH (maseni omjer prema suhoj tvari biomase kvasca) tijekom 15 minuta pri 45–80 °C (svakih 5 °C). Tijekom postupka ekstrakcije mjereni su gubici proteina i suhe tvari biomase te smanjenje koncentracije RNA. Uz relativno malu koncentraciju lužine (10 % NH_4OH), pri 60 °C, postignuto je smanjenje masenog udjela RNA na 1,73 % (prema suhoj tvari biomase kvasca). Pri tome je, posebice, smanjeno vrijeme ekstrakcije (15 minuta).*

Nakon postupka ekstrakcije RNA je izolirana djelomičnim dvostupnjevitim taloženjem s HCl (1. korak pH = 4,9; 2. korak pH = 2,0). Tim su postupkom dobivene dvije čiste frakcije (taloga) u kojima je ispitivana koncentracija RNA. Prinos RNA (g RNA po g RNA u kvascu) u drugom je talogu bio 72,1 %. Čistoća drugog taloga bila je 69,7 %.

Introduction

Compared with conventional food, microbial biomasses contain high amounts of nucleic acids even when expressed on a protein basis: there are 8–25 g of nucleic acids, mostly RNA, per 100 g of microbial proteins *(1)*. The necessity of reducing the nucleic acid content of single cell protein for human consumption has been adequately summarized *(2,3)*. In a previous paper, we described methods for reducing the RNA content of the yeast *Saccharomyces cerevisiae (4)*.

High nucleic acid content, low cell wall digestibility and low methionine content are three of the most important nutritional limiting factors in the use of yeast for human and/or animal consumption *(1,2,5,6)*.

When discussing the hazards for man from a high nucleic acids consumption, it is usually done in terms of the evident effects from metabolized purines on the plasma and urine levels of uric acid (gout and stones in the urinary tract) *(2)*. However, in the absence of knowl-

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edge, might not also possible negative effects of excessive intakes of pyrimidines have to be considered? Orotic acid, an intermediate in the synthesis of pyrimidine bases, has been suggested as a possible cause of liver necrosis in rats fed by milk powder diets *(7)*. Dietary levels as low as 10 ppm have been reported to affect liver metabolism in rats. Furthermore, will an increased intake of the unusual bases (such as methylated purines and pyrimidines) found in small amounts in yeast and bacterial RNA *(8)* be of any significance? Gehrke *et al. (9)* observed elevations of 1-methylinosine, adenosine and N^2,N^2-dimethylguanosine in the urine of leukemic and breast cancer patients.

Because of the unique blockage in the purine metabolism of man, animal experiments for studies concerning dietary RNA effects, etc., are not directly applicable. Rats can, however, be used after inhibition of uricase with a specific inhibitor *(6)*.

When considering possible methods for the reduction of the nucleic acid content, special attention should be paid to the safety and economy of the process. Some of the methods in the literature seem to be complicated, expensive and hardly applicable on a technical scale. Possible utilization of the hydrolysis products of RNA affects the overall economy of the nucleic acid reduction process. In order to have an integrated process, the extracted RNA may be used as the substrate for the production of flavor enhancers *(10–13)*.

Reduction of nucleic acid is often carried out following the separation of yeast proteins in crude form from the whole cell. Yeast is ruptured by any of the methods described in the literature (high-pressure homogenization, colloid mill, sonic disintegration, freeze-thaw treatment, use of lytic enzymes) or a combination of these methods for maximum recovery *(14)*. Rupture of the cells by any of the procedures listed above results in cellular debris and a soluble cytoplasmic fraction containing a high degree of colloidal matter. Any attempt to precipitate the protein fraction from the soluble fraction would also result in the coprecipitation of nucleic acids. Other practical methods are thus necessary to recover the yeast proteins with the least amount of nucleic acid so that the isolated protein can serve as an acceptable source of protein in human diet *(15)*. Known extraction processes for separation and isolating individual products from complex mixtures of natural materials usually involve the yield-optimized purification of one substance or one class of substances but take no account of changes in the chemical or physical properties of other products present in the overall mixture *(6,16,17)*.

The aim of this work was to establish optimum conditions for maximum removal of RNA content in yeast biomass, using alkali and acid solutions, with minimal protein and biomass losses.

Materials and Methods

Microorganism used

Commercially available fresh baker's yeast, *S. cerevisiae* (28 % dry matter) obtained from »PLIVA- Yeast Division«, Savski Marof was employed. The 40-g yeast block wrapped in waxed paper was kept at 4 °C for no more than 7 days. The RNA and protein content of the cells was, on the average, 6.5 and 49 %, respectively, calculated on dry matter bases. Before the extraction procedure, yeast biomass was suspended in redistilled water (pH = 6.5) and kept 10 min at room temperature (25 °C).

RNA extraction with acid (1M HCl) and alkali (1M NaOH)

10 mL volume of yeast suspensions in redistilled water (20 % dry matter) were heated (60 °C) in a water bath at different pH values (pH = 2.0–11.0). Initially, the pH value was adjusted by adding either 1M HCl or 1M NaOH. After the 15 min of extraction procedure, each suspension was transferred into 30 mL centrifuge tubes, cooled immediately to the room temperature, and centrifuged (Beckman Centrifuge, Model J-21B) at 5000 rpm for 15 min at 25 °C. After centrifugation, the amounts of RNA in precipitates and in supernatants were determined (Fig. 1).

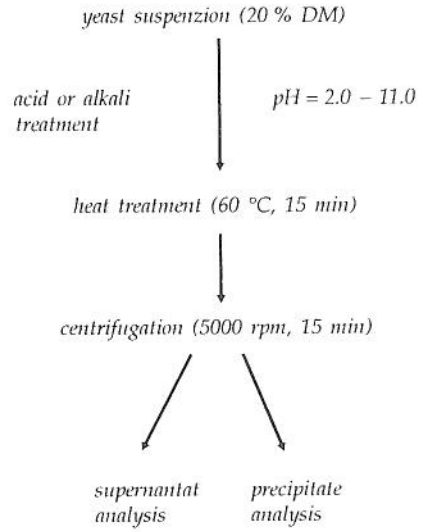

Fig. 1. Scheme of RNA extraction with acid and alkali
Slika 1. Prikaz postupka ekstrakcije RNA kiselinom i lužinom

Thermal treatments with NH_4OH

10 mL volume of yeast suspensions in redistilled water (30 g/L) were put in a Erlenmeyer flasks of 25 mL capacity and homogenized with magnetic stirrer. Extractions with 5, 10 and 15 % of NH_4OH (referring to yeast dry matter) were incubated for 15 minutes at 45–80 °C (step 5 °C). In all cases, the suspension needed 15 min to reach the chosen temperature. After the treatment, samples were transferred into 30 mL centrifuge tubes, cooled immediately under running tap water to the room temperature and centrifuged at 5000 rpm for 15 min at 25 °C. After centrifugation, the amounts of RNA and proteins in supernatants were determined. Precipitates were taken and gravimetric dry matter were assayed.

RNA extraction and precipitation

The experiments with 25 mL of yeast suspensions in redistilled water (30 g/L) were carried out in stirred Erlenmeyer flasks of 100 mL volume. The flasks were submerged in a constant temperature shaking bath (60 °C in Gyrotory Water Bath Shaker Model G76, New Brunswick Sci. Co. Inc.) and the agitation speed was 200 rpm. The suspensions needed 5 min to reach the chosen temperature. RNA was extracted with 10 % NH_4OH (referring to yeast dry matter) for 15 minutes. The pH value of all samples measured at the beginning of the experiment was 10.4. After the extraction procedure samples were cooled immediately under running tap water to the room temperature and centrifuged at 5000 rpm at 25 °C (1^{st} precipitates were the rest yeast biomass). In order to choose an appropriate method to recover the RNA from samples supernatant, two methods were tested:

1) precipitation by adjusting the pH of the extract to pH = 2.0 with HCl. After the centrifugation at 5000 rpm for 15 min at 25 °C, RNA content in the precipitate was determined, and

2) fractional precipitation with HCl until desired pH (pH = 4.6–5.0). After removing the 2^{nd} precipitates (low RNA content, proteins) by centrifugation at 5000 rpm for 15 min at 25 °C, HCl was added to supernatants (residual RNA) until pH = 2.0. At the end of the precipitation process, the 3^{rd} precipitates were obtained (RNA), and

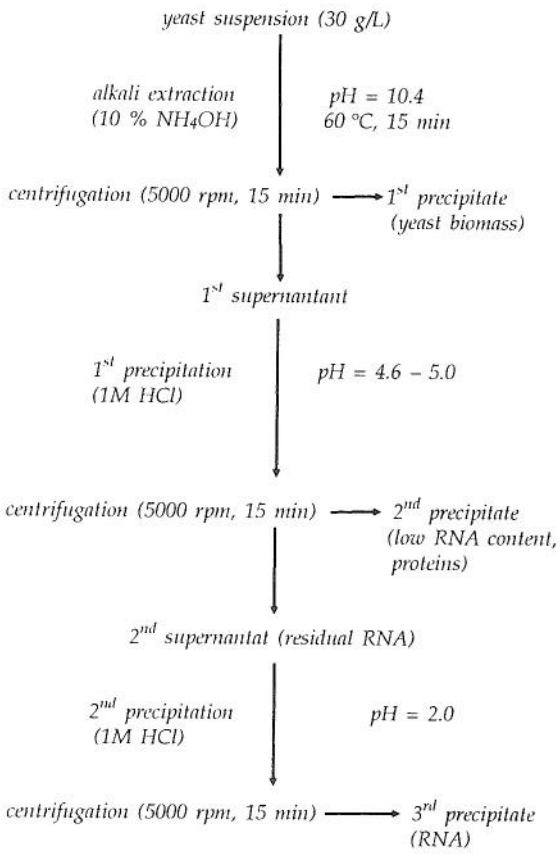

Fig. 2. Scheme of RNA extraction and precipitation
Slika 2. Prikaz postupka ekstrakcije i taloženja RNA

RNA content in 2^{nd} and 3^{rd} precipitate was determined as shown in Fig. 2.

Kinetics of RNA extraction

The experiment with 100 mL of yeast suspension in redistilled water (30 g/L) was carried out in a stirred Erlenmeyer flask of 250 mL volume, submerged in a constant temperature shaking bath (60 °C). The suspension needed 5 min to reach the chosen temperature. RNA was extracted with 10 % NH_4OH (referring to yeast dry matter) for 30 min (the agitation speed was 200 rpm). The pH value of sample measured at the beginning of the experiment was 10.4. Every 5 min of extraction procedure, 5 mL of suspension was pipetted into 10 mL graduated centrifuge tube, cooled immediately under running tap water to the room temperature and centrifuged at 5000 rpm for 15 min at 25 °C. After centrifugation, pH and RNA content (A_{260} values) of sample were determined.

Analytical determinations

Gravimetric dry matter

Yeast suspension (10 mL) was centrifuged at 3000 rpm for 15 minutes, washed twice with physiological solution (1 % of NaCl) and dried at 60 °C for 1 hour, then at 105 °C to constant weight.

Total nucleic acid content

The total nucleic acid content was determined by a simplified spectrophotometric method, based on removal of nucleic acid with 0.5 M $HClO_4$ at 90 °C for 20 min and measuring the absorbance of supernatant at 270 and 290 nm *(18)*.

RNA content

The amounts of RNA in precipitates and in supernatants were determined by measuring absorbances at 260 and 280 nm, so that corrections could be made for protein calculations. An absorptivity of 27 mL/mg cm was used for calculations. In addition, a standard curve was constructed using yeast RNA (Sigma Co.).

Protein content

Lowry's method was used to determine the soluble protein content in precipitates and in supernatants *(19)*. Bovine serum albumin (Fluka) was used to prepare the standard curve.

Results and Discussion

Influence of pH value

Fig. 1. shows that the ribonucleic acid (RNA) content of yeast cells was lowered either at high or at low pH values. The RNA mass fraction of whole cells was 6.5 %. In the range studied, maximum reduction was observed at pH = 2.0. The RNA content of the cells was lowered from 6.5 to 1.2 %, as the pH approached 2.0 by the addition of 1M HCl. The RNA content was lowered to 1.72 % as the pH was raised to 11.0 by the addition of 1M NaOH. Changes in RNA and protein content in acid and alkali heat-treated samples are shown in Table 1. A slight decrease of protein content was observed in all

Table 1. Content of RNA, protein and biomass before and after the extraction treatment
Tablica 1. Maseni udjel RNA, proteina i suhe tvari biomase prije i nakon postupka ekstrakcije

Sample (pH value)	w (proteins) %	w (RNA) %	reduction of w (RNA) %	w (RNA)/w (proteins)	recovery (dry weight) %	recovery (proteins) %
0 (Control)	49.00	6.50	0.00	0.133	100.0	100.0
1 (2)	39.78	1.20	81.54	0.030	66.2	81.2
2 (4)	35.33	2.79	56.99	0.079	63.8	72.1
3 (5)	33.03	3.26	49.82	0.099	58.9	67.4
4 (7)	39.05	2.52	61.29	0.065	74.1	79.7
5 (9)	41.75	2.06	68.46	0.049	77.3	85.2
6 (11)	43.61	1.72	73.58	0.039	80.2	89.0

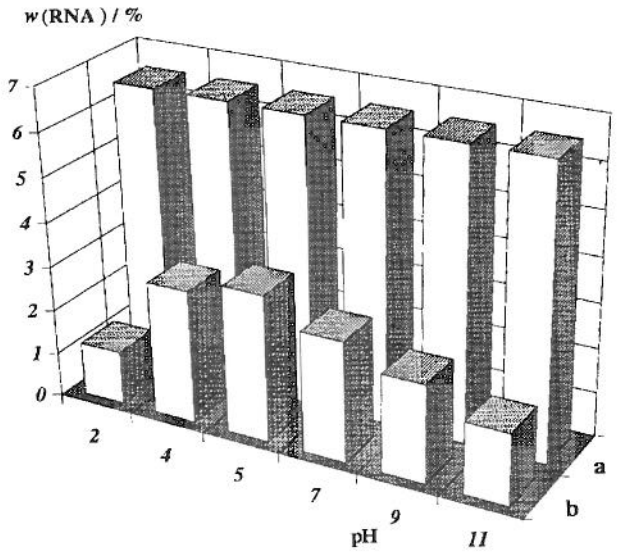

Fig. 3. Influence of pH values on RNA extraction
a – before the extraction
b – after the extraction
Slika 3. Utjecaj pH-vrijednosti na uspješnost ekstrakcije RNA
a – prije ekstrakcije
b – nakon ekstrakcije

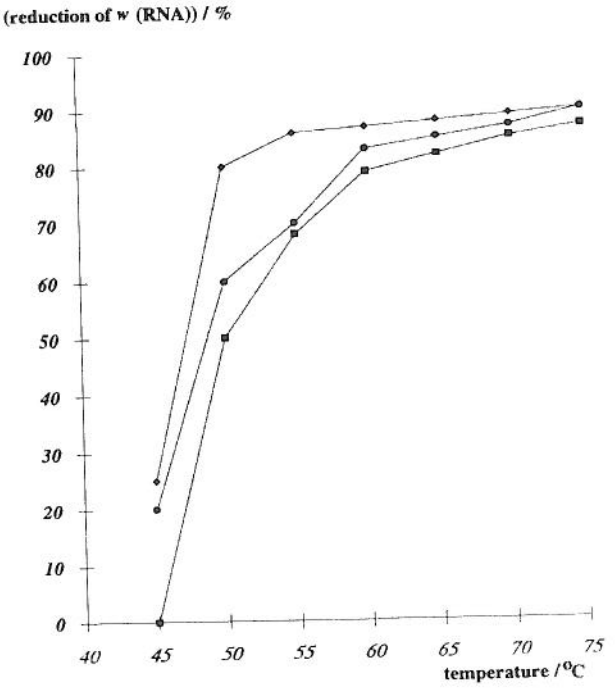

Fig. 4. Influence of incubation temperature on RNA content reduction in the fresh baker's yeast biomass at different concentrations of NH_4OH (♦ 15 % NH_4OH; ● 10 % NH_4OH; ■ 5 % NH_4OH)
Slika 4. Utjecaj temperature inkubacije na smanjenje koncentracije RNA iz biomase svježeg pekarskog kvasca pri različitim koncentracijama NH_4OH (♦ 15 % NH_4OH; ● 10 % NH_4OH; ■ 5 % NH_4OH)

cases. The mass fraction of proteins, which initially was as high as 49 %, decreased to 39.78 % in the case of the acidic treatment (pH = 2.0) and to 43.61 % in the case of alkaline treatment (pH = 11.0). Upon homogenization and centrifugation, much of the RNA remained in the soluble fraction. In all samples, RNA reductions were substantial (49.82 – 81.54 %). Nevertheless, it should be pointed out that RNA reduction on a dry weight basis is a sum of: 1. actual reductions of RNA, and 2. apparent increases of RNA due to relative changes in composition of sample from loss of other solids *(16)*. Most can be seen from changes in the RNA / protein ratio. The RNA/protein mass ratio was lowered from 0.133 to 0.030 after the extraction procedure with acid (pH = 2.0) and to 0.039 after the alkaline treatment (pH = 11.0). Those results point out success of extraction treatments because the recovery of proteins (referring to initial protein content) was 91.2 % and 89 %, respectively. Furthermore, after the extraction with alkali (pH = 11.0), recovery of yeast dry matter (referring to initial yeast dry matter) was 80.2 %. In general, it appears that most pH values investigated reduced RNA enough to make possible increased consumption of yeast without the risk from uric acid toxicity. Dry matter recoveries exceeded 65 % in most cases. Exceptions were in acid heat-treated samples pH = 4.0 (63.8 %) and pH = 5.0 (58.9 %). This also suggests the necessity for a proper balance in the variables, in order to obtain adequate reduction in nucleic acid levels with minimal losses of protein and biomass.

Influence of alkali and temperature

As can be observed in Figs. 2–4, removal of nucleic acids and biomass losses increased with temperature. It is significant that the highest protein losses were obtained between 50 and 55 °C, probably indicating that protease reached maximal activity in this temperature range *(20)*. This effect was enhanced at higher ammonia concentrations, suggesting that the temperature should

Table 2. Yield and purity of precipitated RNA extracted with 10 % NH_4OH at 60 °C, 15 min
Tablica 2. Prinos i čistoća istaložene RNA ekstrahirane s 10 % NH_4OH pri 60 °C, 15 min

Precipitation method	Yield/%	Purity of precipitate/%	
1) HCl until pH = 2.0	68.8	23	
2) Precipitation until pH	Yield in second precipitate/%* pH = 2.0	Purity of precipitate/%**	
		First precipitate	Second precipitate
5.0	62.3	2.4	49.1
4.9	72.1	11.5	69.7
4.8	38.2	18.7	51.1
4.6	23.4	18.2	40.9

* Yield = *m* (RNA obtained) / *m* (RNA in yeast)
** Purity = *m* (RNA in precipitate) / *m* (precipitate obtained)

increase to 60 °C as quickly as possible, in order to minimize the activity of these enzymes. In the experiments with 10 % NH_4OH at 60 °C (Fig. 2.) a reduction in RNA content of 73 % with 10 % protein (Fig. 3.) and 17 % biomass (Fig. 4.) losses were observed. This leads to a final RNA content of nearly 1.7 % of the biomass. A higher incubation temperature (65–80 °C) did not notably increase the reduction of RNA levels, but protein and biomass losses increased considerably, showing that the treatment was severe and that cellular integrity was destroyed. Therefore, incubation temperatures above 60 °C seem unsuitable, since the protein and biomass losses were higher, while the increase in RNA reduction was negligible.

RNA extraction and precipitation

Table 2. shows the results for RNA recovery from the ammonia extraction liquid (pH = 10.4). The RNA precipitation by lowering pH to pH = 2.0 due to addition of HCl produced low RNA purities (23 %) due to the simultaneous protein precipitation *(10)*. Fractionated precipitation might help to overcome this problem by separating the precipitate in two fractions, one with a low RNA content and a second one where mostly RNA is recovered. A satisfactory separation can be obtained if the fractionation pH is accurately established. As can be seen from the data obtained, the pH value for the 1st precipitation has to be fixed at pH = 4.9. After the second precipitation (pH = 2.0), the RNA yield was 72.1 % with a purity of 69.7 %.

Kinetics of RNA extraction

The rate of RNA extraction with 10 % NH_4OH (referring to yeast dry matter) at 60 °C is shown at Fig. 5. Although the extraction process was carried out for 30 min, after 15 minutes the RNA was mostly extracted. The results show that evolution of the extraction can be followed by measuring pH values and absorption at 260 nm wavelength. As can be seen in Fig. 6., the decrease in pH value corresponds to an increase in absorption when extraction takes place. Both the pH values and absorbance are stabilized after 15 minutes and that can be used to determine the extraction end point.

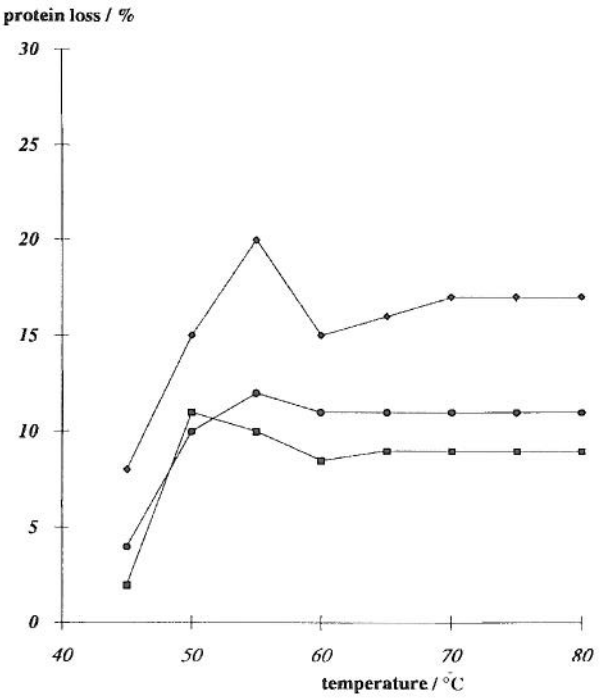

Fig. 5. Influence of incubation temperature on protein loss in fresh baker's yeast biomass at different concentrations of NH_4OH (♦ 15 % NH_4OH; ● 10 % NH_4OH; ■ 5 % NH_4OH)
Slika 5. Utjecaj temperature inkubacije na gubitak proteina iz biomase svježeg pekarskog kvasca pri različitim koncentracijama NH_4OH (♦ 15 % NH_4OH; ● 10 % NH_4OH; ■ 5 % NH_4OH)

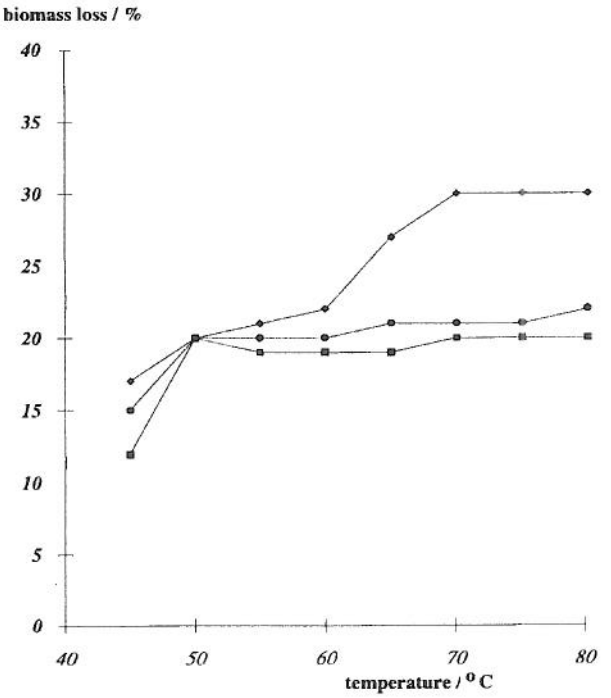

Fig. 6. Influence of incubation temperature on biomass loss in fresh baker's yeast biomass at different concentrations of NH_4OH (♦ 15 % NH_4OH; ● 10 % NH_4OH; ■ 5 % NH_4OH)
Slika 6. Utjecaj temperature inkubacije na gubitak suhe tvari biomase svježeg pekarskog kvasca pri različitim koncentracijama NH_4OH (♦ 15 % NH_4OH; ● 10 % NH_4OH; ■ 5 % NH_4OH)

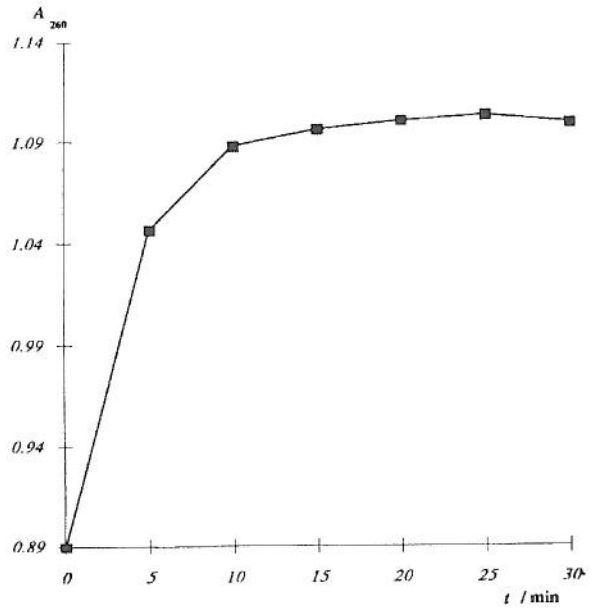

Fig. 7. Kinetic of RNA extraction
Slika 7. Kinetika ekstrakcije RNA

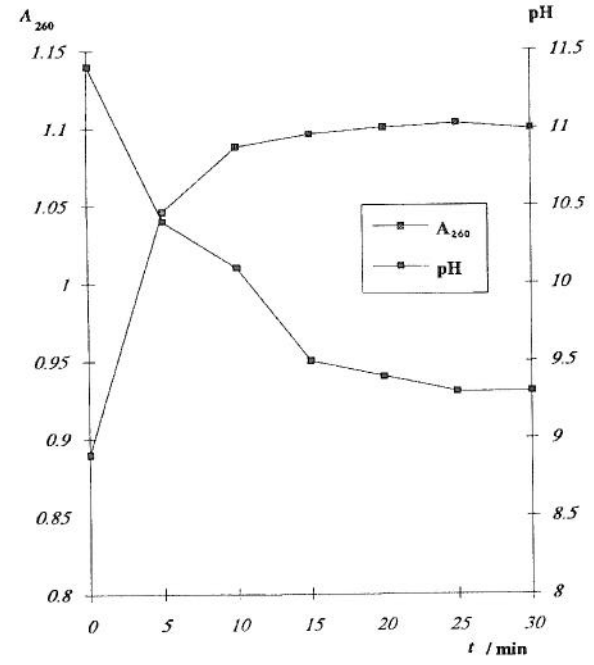

Fig. 8. pH and absorbance (A_{260}) profiles during the RNA extraction (10 % NH_4OH, 60 °C) from the fresh baker's yeast biomass
Slika 8. Prikaz odnosa pH-vrijednosti i apsorbancije (A_{260}) tijekom ekstrakcije RNA (10 % NH_4OH, 60 °C) iz biomase svježeg pekarskog kvasca

Conclusion

Known extraction procedures for separating and isolating individual products from complex mixtures of natural materials usually involve the yield-optimized purification of the substance or one class of substances, but take no account of changes in the chemical or physical properties of other products present in the overall mixture.

The treatment with NH_4OH shows several advantages in relation to other chemical agents. With relatively low concentration (10 %) and moderate temperature of extraction (60 °C) in only 15 minutes, a reduction of 74 % RNA with 11 % of protein and 20 % of biomass losses were obtained.

After the extraction of RNA from the yeast biomass and two-step precipitation and separation (pH = 4.9 and pH = 2.0, respectively), a final extracted RNA content in precipitate was 1.73 % with purity of 69.7 %.

Thus prepared RNA sample could be further hydrolyzed to 5'-ribonucleotides, which would significantly improve economy of the extraction process. In addition, the use of ammonia is more suitable from the point of view of its elimination and/or utilization of residues after the treatment.

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