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# Cultivation of Yeast *Candida tropicalis* 212 and *Candida utilis* 49 in Alfalfa Juice

# Uzgoj kvasaca *Candida tropicalis* 212 i *Candida utilis* 49 u soku lucerne

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*Summary*

*Alfalfa juice, the waste product in green mass processing was investigated as nutritive medium for the cultivation of yeasts as an animal feed supplement. The nutritive medium composition was optimized by addition of molasses which was diluted by juice as substitute for water. Both investigated yeasts* ***Candida tropicalis*** *212 and* ***Candida utilis*** *49 exhibited similar growth and substrate assimilation kinetics* $\mu = 0.228\ h^{-1}$, $Y_{x/s} = 0.614\ g\ g^{-1}$). *To achieve better productivity as well as economy of* ***Candida*** *yeasts biomass cultivation, the experiments were carried out without steam sterilization of the nutritive medium. It was only disinfected by sodium hypochlorite* ($\gamma = 2\ g\ L^{-1}$). *The results obtained indicate the possibility of a relatively simple and economically favorable production of yeasts biomass on the location of alfalfa plant processing.*

*Sažetak*

*Sok lucerne, otpadni proizvod pri obradbi zelene biljne mase, ispitan je kao hranidbena podloga za uzgoj krmnih kvasaca iz roda* ***Candida****. Sastav je hranidbene podloge optimiran dodatkom melase, koja je umjesto vodom razrjeđivana sokom lucerne. Oba ispitivana kvasca* ***Candida tropicalis*** *212 i* ***Candida utilis*** *49 pokazala su vrlo slične kinetičke zakonitosti rasta i asimilacije supstrata* ($\mu = 0{,}228\ h^{-1}$, $Y_{x/s} = 0{,}614\ g\ g^{-1}$). *Da bi se postigla bolja ekonomičnost procesa uzgoja kvaščeve biomase, provedeni su i pokusi u toplinski nesterileziranoj hranidbenoj podlozi. Hranidbena je podloga dezinficirana samo s otopinom natrij-hipoklorita* ($\gamma = 2\ g\ L^{-1}$). *Dobiveni rezultati upućuju na mogućnost relativno jednostavnog i ekonomičnog procesa proizvodnje kvaščeve biomase na mjestu dobivanja soka lucerne.*

## Introduction

The increase in human population causes an always greater deficiency of protein food.

The FAO experts have estimated that deficiency of animal proteins would increase up to 18 million tons until 2000 year. According to the calculation of future development of cattle-breeding it is necessary to ensure 2 million tons of proteins per year from other sources. This defect could be compensated by cultivation of soya on 40 million hectare ploughland but this is practically impossible to realize (*1*).

Therefore, microbial technology could play an important role. According to present development of science and technology it might be possible to produce this mass of proteins as microbial biomass in 2000 bioreactors of 200 $m^3$ volumes each, occupying only 1 hectare of ploughland (*2*). Investigations of production of Single-cell protein (SCP) cultivated on cheap substrates include nearly all microbial species: algae (*3*), actinomycetae (*4,5*), bacteria (*3,6*), yeasts (*7–10*), moulds and mushrooms (*11–13*).

The fact that microbial cells can grow on different waste materials is very important for our environment. Problems of the pollution of waste water and other industrial residues could be solved by the employment of microorganisms (*14,15*). From the aspect of animal food applications the cultivation of alfalfa plants is interesting because of the large contribution of green mass, high nutritive value and low cost of production. In recent years the carbohydrate refuse from farms (*16*) as well as waste water from the alfalfa processing are also interesting as cheap substrates for microorganisms cultivation. The production of alfalfa, in favorable conditions, gives more protein per unit surface compared to other feed plants. The alfalfa can be used as fresh green mass, quality hay and silage. Also, alfalfa is used for industrial processing in the alfalfa flour, brickets and pellets as effi-

cient additive for equalizing protein component of feed concentrates (*17*). In highly developed countries industrial processes for the alfalfa production include a fresh green juice, which is obtained by pressing the plant. Proteins are separated from the fresh green juice by thermic coagulation (80–100 °C) and subsequent filtration. These proteins are valuable and usable for feed composition enrichment (*18,19*). The residual deproteinized juice can be used as fertilizer in liquid form due to sufficient potassium participation (*20*). Also, the deproteinized juice can be used as nutritive medium for cultivation of microorganisms in production of cheap high protein additives to non-ruminant feed with considerable reduction BOD/COD value.

Published data on usage of the deproteinized alfalfa juice as substrate for microorganisms cultivation are rather rare. Only few papers refer to yeasts as microbial species which could satisfy demands for biomass production in the deproteinized waste alfalfa juice (*18,21,22*).

This paper deals with usage possibilities of the alfalfa green juice as substrate for the cultivation of *Candida* yeast for obtaining rich protein feed preparates. The process of yeast biomass cultivation with respect to increased productivity as well as the solution of the problem of waste water from the alfalfa processing have been studied.

## Materials and methods

*Microorganisms*: The both yeasts *Candida tropicalis* 212 and *Candida utilis* 49 have been taken from the Collection of microorganisms, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb.

*Preparation of the alfalfa juice*: The alfalfa plants were obtained from experimental fields at Botinec, Institute of plant breeding and production, Zagreb. The same day after swath, the chopping by the chopping machine, »Lifam« – Stara Pazova (15KW, 220V), as well as pressing by the hydraulic press made at the Food Technology and Biotechnology Faculty of Zagreb workshop were performed. The pressed juice was frozen (–18 °C), homogenized and analyzed, before usage for preparation of nutritive media (Table 1).

Table 1. Composition of the alfalfa juice and molasses
Tablica 1. Sastav soka lucerne i melase

| Composition* / Sastav* | Molasses / Melasa % | Alfalfa juice / Sok lucerne % |
|---|---|---|
| Dry matter / Suha tvar | 77 | 6.12 |
| Anorganic matter / Anorganska tvar | 13 | 16.50 |
| Organic matter / Organska tvar | 81 | 83.50 |
| Sugars / Šećeri | 50 | 22.22 |
| Proteins / Proteini | 8 | 35.46 |

*Results expressed on dry matter
Rezultati izraženi na suhu tvar

*Molasses*: The molasses used for preparation of nutritive media was supplied from sugar refinery in Virovitica. The quality of molasses composition have been as satisfactory as asked proposition (Table 1).

*Nutritive media*: Media for yeasts cultivation contained the alfalfa juice and the alfalfa juice enriched with molasses in concentration range from 20–160 g $L^{-1}$ (from 10 to 80 g $L^{-1}$ sugar from molasses). All nutritive media were set up with 1 mol $L^{-1}$ $H_2SO_4$ at pH value 4.5. The media were sterilized for 30 min at 121 °C, and at 1 bar pressure, except in experiments of batch cultivation which were performed in partially aseptic conditions. Sodium hypochlorite ($\gamma$ = 2 g $L^{-1}$ with 13 % active chlorine) was added to these nutritive media.

*Cultivation*: All preliminary experiments were carried out in Erlenmeyer's flasks of 500 mL volume with 100 mL nutritive medium on a rotary shaker (200 rpm) during 24 hours at 28 °C. During cultivation time pH value was not corrected. Batch cultivations were carried out in 3 L nutritive medium in the laboratory bioreactor (volume 7 L) PEC – fermenter, Chemap AGE, Switzerland. In all experiments performed in the bioreactor, the temperature and pH value were regulated automatically (temperature from 28 to 30 °C, pH value from 4.3 to 4.6). Mixing and air flow for aeration were performed in a way that the oxygen saturating concentration was not less than 20 %.

*Inoculum*: The inoculum for all cultivations was prepared in Erlenmeyer's flasks (volume 500 mL) in 100 mL medium (the alfalfa juice). Cultivation of the inoculum was carried out on a rotary shaker (200 rpm) during 24 hours at 28 °C. During the cultivation time the pH value of inoculum was not corrected. Nutritive media for the cultivation were inoculated with volume ratio of 10 % (1.5 to 2.2 g $L^{-1}$ dry matter) of prepared inoculum.

### *Analytical methods*

*Dry matter* of yeast biomass was determined in aliquot of culture by centrifugation at 5000 rpm during 15 min. The sediment of biomass was washed out by distilled water and dried at 105 °C till constant mass.

*Kinetics of sugar consumption* was followed by the RS method (23) after the hydrolysis of supernatant components by an acid.

*Ammonium nitrogen* was determined by titration after distillation from a supernatant in the Parnas – Wagner instrument.

*Chemical oxygen demand* (COD) was determined by oxidation of organic matter present in juice (*24*). The data were used for calculation of a substrate transformation yield into a biomass.

*Transformation yield factor* $Y_{x/s}$ was calculated by equation

$$Y_{x/s} = \Delta x / \Delta s \qquad /1/$$

where $\Delta x = \gamma$ (Biomass, d.w)$_t$ – $\gamma$ (Biomass, d.w)$_o$ /2/

$$\Delta s = \gamma \text{ (Substrate)}_t - \gamma \text{ (Substrate)}_o \qquad /3/$$

$\gamma$ (Biomass, d.w.)$_o$ = concentration of biomass dry matter at the beginning of cultivation

$\gamma$ (Biomass, d.w.)$_t$ = concentration of biomass dry matter at the end of cultivation

$\gamma$ (Substrate)$_o$ = beginning substrate concentration expressed as total organic matter determined as COD value $\gamma$ ($O_2$)

$\gamma$ (Substrate)$_t$ = substrate concentration at the end of cultivation expressed as total organic matter determined as COD value $\gamma$ ($O_2$)

For the yield factor calculation organic matter was not determined directly. It was supposed that COD value of the nutritive medium at the beginning and the end of yeast cultivation corresponded to the organic matter concentration in medium.

## Results and discussion

The alfalfa juice prepared by pressing alfalfa plants satisfies basic needs for growth and propagation of the yeasts *Candida tropicalis* 212 and *Candida utilis* 49. This is evident from the results shown in Table 2 and Table 3. Relatively low values of the process productivity (Pr = 0.56 g $L^{-1}h^{-1}$) and the low increase of the biomass dry matter ($\Delta X$ = 11.70 g $L^{-1}$) obtained for the yeast *C. tropicalis* 212 (Table 2), and for the yeast *C. utilis* 49 (Pr = 0.73 g $L^{-1}h^{-1}$ and $\Delta X$ = 14.55 g $L^{-1}$) (Table 3), indicated that production of the yeast biomass in the alfalfa juice would not be lucrative economically.

Enrichment of the alfalfa juice by molasses was used for greater increase of yeast biomass and for shortening the process. Addition of complex media such as molasses and corn steap liquor (CSL) for enrichment of nutritive medium, was successfully used by others (*10*). Also, it was tried to establish whether the alfalfa juice may be used as dilution agent for molasses instead of water as in classical production of yeast biomass. The yield of biomass dry matter for both investigated yeasts proportionally rises by addition of molasses in the alfalfa juice. That is shown by the obtained results in Tables 2, and 3. So, by an enrichment of the alfalfa juice with 2–12 % of molasses the increase of a biomass dry matter of *C. tropicalis* 212 was greater for 4.25–24.7 g $L^{-1}$ (Table 2). However by addition of 16 % of molasses into alfalfa juice proportional increase of the yeast biomass *C. tropicalis* 212 cannot be achieved. It is evident that at low concentration of added molasses the complete transformation of the alfalfa juice and molasses components into biomass occurred, while at higher concentration this tran-

Table 2. Comparison of success indicators of cultivation[1] of yeast *C. tropicalis* 212 in alfalfa juice enriched with molasses$_{50}$

Tablica 2. Usporedba pokazatelja uspješnosti procesa uzgoja[1] kvasca *C. tropicalis* 212 u soku lucerne obogaćenom melasom$_{50}$

| Mass fraction of added molasses$_{50}$ in medium /%<br>Maseni udio dodane melase$_{50}$ u podlogu /% | 0 | 2 | 4 | 8 | 12 | 16 |
|---|---|---|---|---|---|---|
| Time (t) /h<br>Vrijeme (t) /h | 21 | 21 | 24 | 24 | 24 | 24 |
| Increase in biomass dry matter ($\Delta X$) /g $L^{-1}$<br>Prirast suhe tvari biomase ($\Delta X$) /g $L^{-1}$ | 11.70 | 15.95 | 22.30 | 28.00 | 36.40 | 33.20 |
| Substrate consumption ($\Delta S$) /g $L^{-1}$<br>Utrošeni supstrat ($\Delta S$) /g $L^{-1}$ | 24.12 | 30.67 | 42.56 | 49.82 | 58.90 | 64.09 |
| Yield factor ($Y_{X/S}$) /g $g^{-1}$<br>Stupanj pretvorbe supstrata ($Y_{X/S}$) /g $g^{-1}$ | 0.485 | 0.520 | 0.524 | 0.562 | 0.618 | 0.516 |
| Process productivity (Pr) / g $L^{-1}h^{-1}$<br>Produktivnost procesa (Pr) / g $L^{-1}h^{-1}$ | 0.56 | 0.70 | 0.93 | 1.17 | 1.52 | 1.38 |
| Specific growth rate ($\mu_{max}$) / $h^{-1}$<br>Specifična brzina rasta ($\mu_{max}$) / $h^{-1}$ | 0.078 | 0.103 | 0.105 | 0.110 | 0.123 | 0.116 |

[1]Cultivation on shaker

Cultivation conditions: 200 rpm, 28 °C, $\gamma$ (inoculum) = 1.50–2.20 g $L^{-1}$ d.w., pH = 4.5

[1]Uzgoj na tresilici

Uvjeti uzgoja: 200 rpm, 28 °C, $\gamma$ (cjepivo) = 1,50–2,20 g $L^{-1}$ s.t., pH = 4,5

Table 3. Comparison of success indicators of cultivation[1] of *C. utilis* 49 in alfalfa juice enriched with molasses$_{50}$

Tablica 3. Usporedba pokazatelja uspješnosti procesa uzgoja[1] kvasca *C. utilis* 49 u soku lucerne obogaćenom melasom$_{50}$

| Mass fraction of added molasses$_{50}$ in medium /%<br>Maseni udio dodane melase$_{50}$ u podlogu /% | 0 | 2 | 4 | 8 | 12 | 16 |
|---|---|---|---|---|---|---|
| Time (t) /h<br>Vrijeme (t) /h | 20 | 20 | 24 | 24 | 24 | 24 |
| Increase in biomass dry matter ($\Delta X$) /g $L^{-1}$<br>Prirast suhe tvari biomase ($\Delta X$) /g $L^{-1}$ | 14.55 | 18.50 | 23.69 | 28.78 | 35.76 | 37.11 |
| Substrate consumption ($\Delta S$) /g $L^{-1}$<br>Utrošeni supstrat ($\Delta S$) /g $L^{-1}$ | 30.56 | 37.60 | 45.55 | 49.96 | 57.95 | 61.54 |
| Yield factor ($Y_{X/S}$) /g $g^{-1}$<br>Stupanj pretvorbe supstrata ($Y_{X/S}$) /g $g^{-1}$ | 0.476 | 0.492 | 0.520 | 0.576 | 0.617 | 0.603 |
| Process productivity (Pr) / g $L^{-1}h^{-1}$<br>Produktivnost procesa (Pr) / g $L^{-1}h^{-1}$ | 0.73 | 0.77 | 0.99 | 1.20 | 1.49 | 1.55 |
| Specific growth rate ($\mu_{max}$) / $h^{-1}$<br>Specifična brzina rasta ($\mu_{max}$) / $h^{-1}$ | 0.119 | 0.129 | 0.118 | 0.125 | 0.134 | 0.135 |

[1]Cultivation on shaker

Cultivation conditions: 200 rpm, 28 °C, $\gamma$ (inoculum) = 1.50–2.20 g $L^{-1}$ d.w., pH = 4.5

[1]Uzgoj na tresilici

Uvjeti uzgoja: 200 rpm, 28 °C, $\gamma$ (cjepivo) = 1,50–2,20 g $L^{-1}$ s.t., pH = 4,5

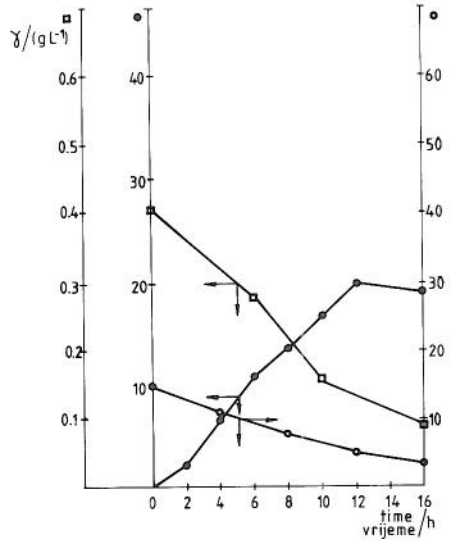


Fig. 1. Batch cultivation of yeast *C. tropicalis* 212 in alfalfa juice. • – increase of biomass dry matter γ/ g $L^{-1}$; □ – ammonium nitrogen γ/ g $L^{-1}$; ∘ – sugar consumption γ/ g $L^{-1}$.

Slika 1. Šaržni uzgoj kvasca *C. tropicalis* 212 u soku lucerne. • – prirast suhe tvari biomase γ/ g $L^{-1}$;□– amonijačni dušik γ/ g $L^{-1}$; ∘ – potrošnja šećera γ/ g $L^{-1}$.

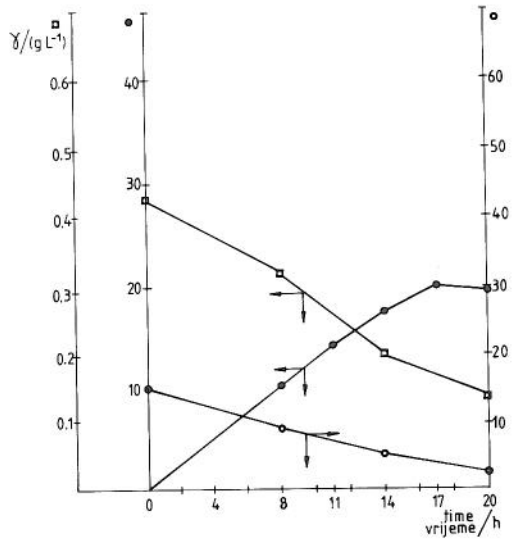


Fig. 2. Batch cultivation of yeast *C. utilis* 49 in alfalfa juice. • – increase of biomass dry matter γ/ g $L^{-1}$; □ – ammonium nitrogen γ/ g $L^{-1}$; ∘ – sugar consumption γ/ g $L^{-1}$.

Slika 2. Šaržni uzgoj kvasca *C. utilis* 49 u soku lucerne. • – prirast suhe tvari biomase γ/ g $L^{-1}$; □ – amonijačni dušik γ/ g $L^{-1}$; ∘ – potrošnja šećera γ/ g $L^{-1}$.

sformation is not complete. This could be due to the substrate inhibition, but it is more plausible that the Crabtree effect becomes expressive at higher sugar concentrat-

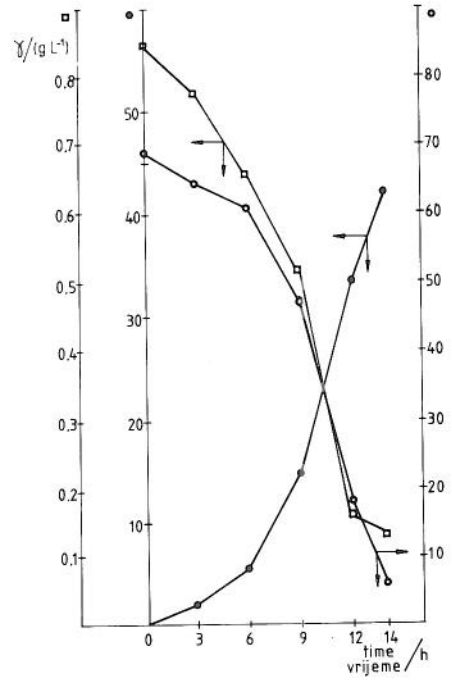


Fig. 3. Batch cultivation of yeast *C. tropicalis* 212 in alfalfa juice enriched with 12 % of $molasses_{50}$. • – increase of biomass dry matter γ/ g $L^{-1}$; □ – ammonium nitrogen γ/ g $L^{-1}$; ∘ – sugar consumption γ/ g $L^{-1}$.

Slika 3. Šaržni uzgoj kvasca *C. tropicalis* 212 u soku lucerne obogaćenom s 12 % $melase_{50}$. • – prirast suhe tvari biomase γ/ g $L^{-1}$; □ – amonijačni dušik γ/ g $L^{-1}$; ∘ – potrošnja šećera γ/ g $L^{-1}$.

ion. Therefore a part of sugar can be fermented into ethanol. Similar results were obtained also with the yeast *C. utilis* 49 (Table 3). The optimization of nutritive media composition was carried out in experiments of yeast cultivation in Erlenmeyer's flasks on a rotary shaker.

The next step was the defining of process parameters in a laboratory bioreactor under controlled conditions, the most important being the supplying of yeast cells with oxygen. It is known that oxygen from air plays a role of hydrogen acceptor in yeast propagation in aerobic conditions. The hydrogen originated from substrate degradation. The demand for oxygen depends on the kind of carbon source which is used as an energy source (*25*).

The results of batch cultivation of the yeasts *C. tropicalis* 212 and *C. utilis* 49 carried out in a bioreactor show that it is possible to improve the results of the process by control and regulation of the process parameters (i.e. pH, temperature, oxygen supply). The advantage of the fermentation process in a bioreactor over that in shake flask cultures is reflected through shorter cultivation time and an increase of dry matter of biomass. By cultivation of the yeast *C. tropicalis* 212 in the alfalfa juice in bioreactor (Fig. 1.) the culture reached the stationary state of growth already after 12 hours, while the increase of the yeast biomass was 67 % greater than that achieved in Erlenmeyer's flasks (Table 2.).

With *C. utilis* 49 the results achieved were 35 % greater after 17 hours of cultivation (Fig. 2., Table 3). Figs. 1. and 2. show that in both cases growth is limited by some other factors of nutritive medium rather than by carbon and nitrogen sources. Reducing matters and ammonium nitrogen are not used up at all.

By cultivation of the yeast *C. tropicalis* 212 for 14 hours in the bioreactor (Fig. 3.) in the alfalfa juice enriched by molasses an increase of 42.38 g $L^{-1}$ of the yeast

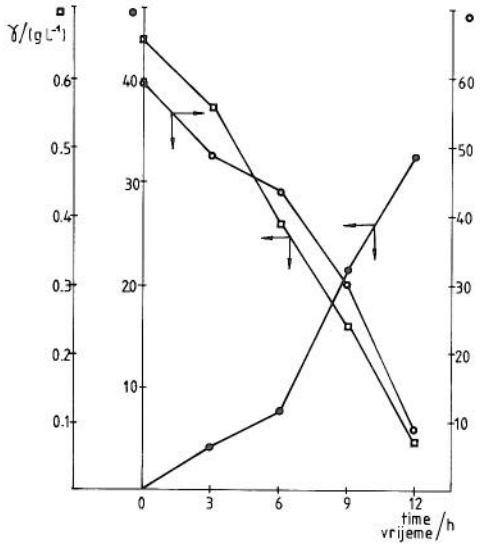


Fig. 4. Batch cultivation of yeast *C. utilis* 49 in alfalfa juice enriched with 10 % of molasses$_{50}$.
• – increase of biomass dry matter γ/ g $L^{-1}$; □ – ammonium nitrogen γ/ g $L^{-1}$; ○ – sugar consumption γ/ g $L^{-1}$.

Slika 4. Šaržni uzgoj kvasca *C. utilis* 49 u soku lucerne obogaćenom s 10 % melase$_{50}$.
• – prirast suhe tvari biomase γ/ g $L^{-1}$; □ – amonijačni dušik γ/ g $L^{-1}$; ○ – potrošnja šećera γ/ g $L^{-1}$.

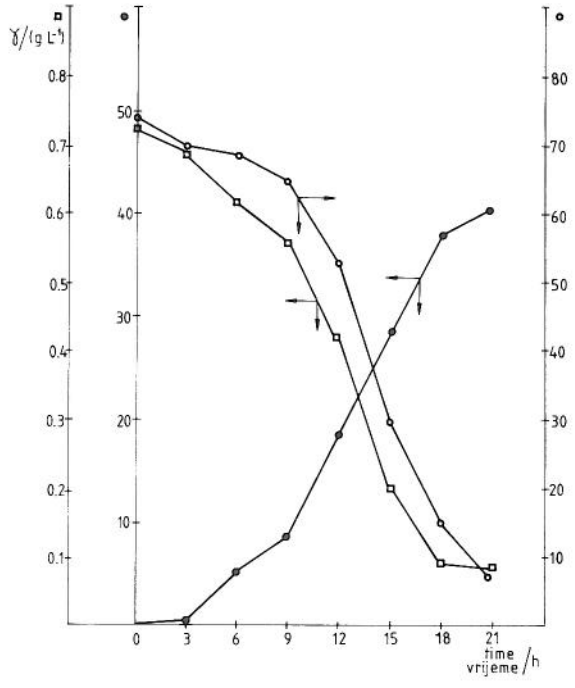


Fig. 5. Batch cultivation of yeast *C. tropicalis* 212 in alfalfa juice enriched with 12 % of molasses$_{50}$ and disinfected by sodium hypochlorite (γ = 2 g $L^{-1}$).
• – increase of biomass dry matter γ/ g $L^{-1}$; □ – ammonium nitrogen γ/ g $L^{-1}$; ○ – sugar consumption γ/ g $L^{-1}$.

Slika 5. Šaržni uzgoj kvasca *C. tropicalis* 212 u soku lucerne obogaćenom s 12 % melase$_{50}$ i dezinficiranom s natrij–hipokloritom (γ = 2 g $L^{-1}$).
• – prirast suhe tvari biomase γ/ g $L^{-1}$; □ – amonijačni dušik γ/ g $L^{-1}$; ○ – potrošnja šećera γ/ g $L^{-1}$.

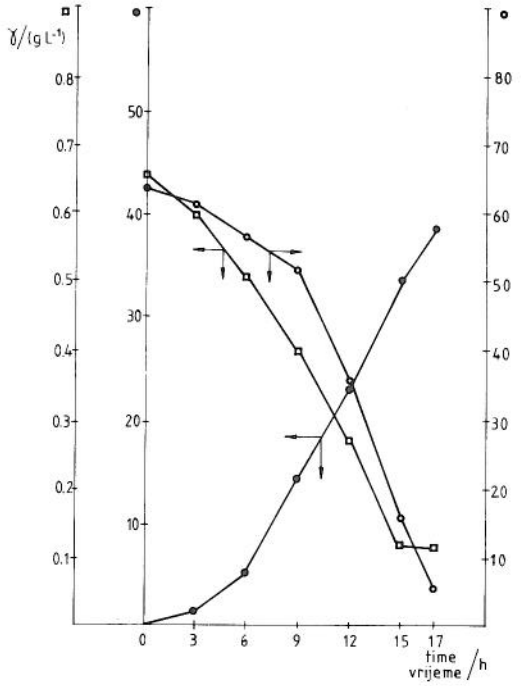


Fig. 6. Batch cultivation of yeast *C. utilis* 49 in alfalfa juice enriched with 10 % of molasses$_{50}$ and disinfected by sodium hypochlorite (γ = 2 g $L^{-1}$).
• – increase of biomass dry matter γ/ g $L^{-1}$; □ – ammonium nitrogen γ/ g $L^{-1}$; ○ – sugar consumption γ/ g $L^{-1}$

Slika 6. Šaržni uzgoj kvasca *C. utilis* 49 u soku lucerne obogaćenom s 10 % melase$_{50}$ i dezinficiranom s natrij-hipokloritom (γ = 2 g $L^{-1}$).
• – prirast suhe tvari biomase γ/ g $L^{-1}$; □ – amonijačni dušik γ/ g $L^{-1}$; ○ – potrošnja šećera γ/ g $L^{-1}$.

biomass was achieved, being twice the increase in juice without molasses addition (Fig. 1.). Similar results were established for the cultivation of yeast *C. utilis* 49 in enriched alfalfa juice (Fig. 4.). In 12 hours the increase of the biomass dry matter obtained was 32.65 g $L^{-1}$, this was also about two times greater than increase achieved in non-enriched juice (Fig. 2.).

During cultivation the presence of ethanol was not established in cultures, and it could be concluded that the Crabtree effect did not occur. Also, data suggest that sugar assimilation was not limited by concentration of dissolved oxygen.

The alfalfa juice can be damaged by original microflora and an addition of chemical disinfectants could be used for temporary juice conservation. Cultivation of yeast biomass in nutritive medium treated with sodium hypochlorite showed the possibility of carrying out of relatively simple and economically favorable process directly at the location where the juice is obtained (e.g. farms, agricultural fields etc.).

The yeast *C. tropicalis* 212 cultivation in partially aseptic conditions in the alfalfa juice enriched by 12 % of molasses (Fig. 5.) during 21 hours, gave an increase of biomass dry matter 40.75 g $L^{-1}$. During 17 hours, 91 % of sugar and about 89 % of ammonium nitrogen were assimilated (Fig. 6.). In comparison with batch cultivations, which were carried out in the nutritive medium

sterilized by steam (Figs. 3., 4.), that process was less successful. The reason for weak yeast growth at the beginning of the process (expressive lag phase of growth for both yeasts), could be considered to be due to reactions of the sodium hypochlorite with some substances from medium and a consequent of rise chemical compounds which partially inhibit the yeast growth. More successful cultivation process in nutritive media sterilized by steam could be explained by heating activity or thermolysis or even enzyme hydrolysis of polymeric molecules (proteins and polysaccharides), which increased their solubility and made them more accessible for yeast cells metabolism.

## Conculsions

The alfalfa juice, as a waste product from the alfalfa plant processing could be used as nutritive medium for cultivation of *Candida* yeasts, well-known as feed yeasts.

The experiments with both investigated yeasts showed an increase of biomass yields in the case when the experiments were carried out in the alfalfa juice enriched by molasses. The addition of about 12 % of molasses (i.e. 6 % of sugar) showed to be optimal one.

It seems also possible to conclude, that in some future commercial process, molasses could be successfully diluted by the alfalfa juice as a substitute for water. In that way, the consumption of water for dilution of molasses in the yeast production could be reduced; the yield of biomass per unit of molasses carbon would be enhanced and, as a consequence, the economy of process would be increased, too.

The process could be carried out without preliminary steam sterilization of the nutritive medium if using sodium hypochlorite as disinfectant.

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