THESES OF DOCTORAL (Ph.D.) DISSERTATION

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OPTIMIZATION OF SINGLE-CELL PROTEIN PRODUCTION USING *KLUYVEROMYCES* STRAINS

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1 INTRODUCTION AND AIM

During manufacture of cheese and quarg products, approximately 970 million dm^3 of sweet and sour whey are produced annually in Hungary. Only a very small proportion of this huge amount of whey is used for animal nutrition, and roughly 50% of the whey produced is used to formulate products, i.e., food and feedstuffs. The remainder is treated as waste. It is sometimes dumped into surface water or sprayed onto fields, thus causing damage to local ecosystems.

Because of high moisture content, it is not economic to transport the whey for drying. In addition, the energy costs of drying are extremely high. As a result, in view of the current sales prices, the manufacture of whey powder is not a profitable activity.

Whey may be used as a valuable raw material for a wide range of applications in the food, pharmaceutical and biogas industries. However, the amount of whey processed worldwide is only about half of the annual global whey output.

An alternative to the traditional uses of whey is the production of single-cell protein (SCP). During manufacture of SCP, whey is inoculated with appropriate yeasts, e.g., *Kluyveromyces* species. The lactose and lactate contents of whey provide nutrients for these microorganisms to grow properly and produce proteins. The finished product, which has a protein content of approximately 50%, is of high biological value (87%). After proper pretreatment, it is utilized in both human and animal nutrition as a food and feed supplement.

Starting in the 1950s, very intensive research activity had been focused on the issues of SCP production but then it largely slowed down due to economic reasons. However, because of the efforts made worldwide to reduce environmental load, the question of whey utilization has recently become a high-priority research topic again.

Although gene technology has opened up new ways in biotechnology, it has not changed the basics of technological processes. The parallel use of genetically engineered novel microbial strains and traditional biotechnological processes is expected in the future despite the fact that some of the inherited traits are not always stable enough.

The primary purpose of this research was to find a yeast strain suitable for use in commercial production of SCP from whey. The strains tested were to be screened for growth characteristics such as maximum viable cell count (N_{max}) and maximum specific growth rate (μ_{max}), therefore, the fermentation parameters providing optimum conditions for the strains to reach N_{max} and μ_{max} were determined first. The glucose, galactose, ethanol, and lactose contents of whey were also quantified during the trials.

The yeast strains were then graded on the basis of their production performance. For this reason, maximum total solids concentration (x_{max}) and yield values such as production rate (dx/dt) and biomass yield ($Y_{x/S}$) were determined under optimum fermentation conditions.

Setting the optimum fermentation parameters determined in this study, together with the use of novel biotechnological procedures, may contribute to the development of an economic SCP production technology.

2 MATERIALS AND METHODS

Based on data reported in the scientific literature, two yeast species (i.e., *Kluyveromyces marxianus* and *K. lactis*) appeared to be viable candidates for use in SCP production from whey. As shown in **Table 1**, five strains belonging to the aforementioned two species were purchased for experimental purposes. All these strains are available in Hungary, with four of them being deposited in a Hungarian culture collection. This fact is thought to be significant in terms of future commercial applications.

Table 1 Yeast strains employed in the experiments

Yeast Strain		Supplier
Kluyveromyces lactis	NCAIM Y 00260	National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary
Kluyveromyces marxianus	NCAIM Y 00933	
Kluyveromyces marxianus (fragilis)	NCAIM Y 00697	
Kluyveromyces marxianus (fragilis)	NCAIM Y 0463	
Kluyveromyces marxianus	LAF 4	Chr. Hansen, Hørsholm Denmark

The experiments were carried out in the accredited Central Laboratory of the Institute of Food Science at the University of West Hungary (microbiological and analytical laboratories).

The fermentation equipment consisted of an automated BIOFLO III® batch/continuous fermenter with a working volume of 1.25 dm^3 (New Brunswick Scientific, Edison, NJ) and an MX3 Biosampler® (New Brunswick). Fermentations were run batchwise for 48 h using sweet whey (pH 6.3, 50 g/dm^3 lactose content) as raw material.

Samples were taken every 4 h and their glucose, galactose, ethanol, and lactose levels were quantitated by high-performance liquid chromatography. Growth parameters such as N_{max} and μ_{max} were also calculated and two strains

were selected for further SCP production trials, in which x_{max}, dx/dt, and $Y_{x/S}$ were determined.

Both sets of experiments were performed in duplicate. The instruments used were calibrated before each measurement session. The data obtained were subjected to one-way and two-way analysis of variance using the general linear model procedure. Significant differences among the means were determined by using Duncan's post-hoc test at $P < 0.05$.

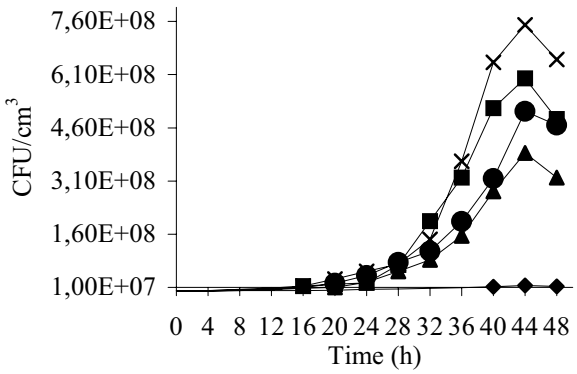
3 RESULTS AND DISCUSSION

3.1 Optimum Fermentation Parameters

Temperature, pH, and agitation were set at 30°C, 4.5, and 300 rpm, respectively, during the trials. Airflow rate was at 0.5, 1.0, 1.5, or 2.0 VVM (air volume/medium volume per min).

3.2 Selection of Strains Based on Their Production Parameters

3.2.1 Maximum viable cell counts (N_{max}) and maximum specific growth rates (μ_{max}) reached by the strains tested



—■—*K. lactis* NCAIM Y 00260* —▲—*K. marxianus* LAF 4*
—x—*K. marxianus* NCAIM Y 00933* —●—*K. marxianus* NCAIM Y 00463*
—♦—*K. marxianus* NCAIM Y 00697* * n = 3

Figure 1 Growth curves of the strains tested

It is illustrated in **Figure 1** that all the *Kluyveromyces* strains tested were measured to produce maximum viable cell counts at h 44. Maximum specific growth rates (μ_{max}) for the particular strains were then calculated from the viable cell counts obtained as shown in **Table 2**.

Table 2 Maximum logarithmic viable cell counts (Log N_{max}) and maximum specific growth rates (μ_{max}) reached by *Kluyveromyces* strains during single-cell protein production

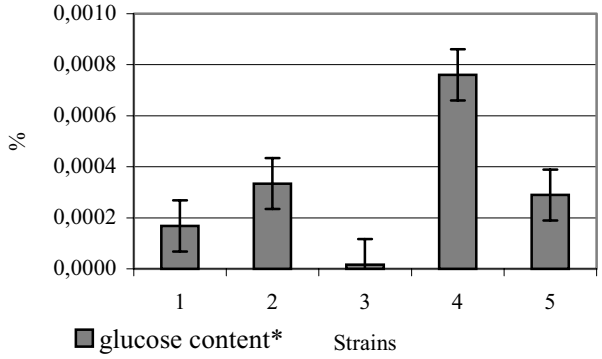
Strain	**Log N_{max}* (Log CFU/ml)**	**μ_{max}* (1/h)**
Kluyveromyces lactis NCAIM Y 00260	8.78 ± 0.04^{a}	0.217 ± 0.014^{a}
Kluyveromyces marxianus LAF 4	8.57 ± 0.15^{b}	0.168 ± 0.006^{b}
Kluyveromyces marxianus NCAIM Y 00933	8.87 ± 0.08^{a}	0.163 ± 0.007^{b}
Kluyveromyces marxianus NCAIM Y 00463	8.67 ± 0.21^{b}	0.163 ± 0.007^{b}
Kluyveromyces marxianus NCAIM Y 00697	7.17 ± 0.10^{c}	0.104 ± 0.007^{c}

* Each value is the mean ± standard deviation of 3 trials.
[abc] Values without a common superscript letter in the same column are significantly different ($P < 0.05$).

As for the maximum viable cell counts, *K. marxianus* LAF 4 and *K. marxianus* NCAIM Y 00463 showed significantly lower values ($P < 0.05$) than did *K. marxianus* NCAIM Y 00933 or *K. lactis* NCAIM Y 00260. However, *K. marxianus* NCAIM Y 00697 produced the poorest result in this respect of all the *Kluyveromyces* strains tested.

The maximum specific growth rate of *K. lactis* NCAIM Y 00260 was significantly higher ($P < 0.05$), whereas the μ_{max} of *K. marxianus* NCAIM Y 00697 was found to be significantly lower ($P < 0.05$) than the values reached by any of the other three strains.

3.3 Glucose Content of the Fermentation Medium

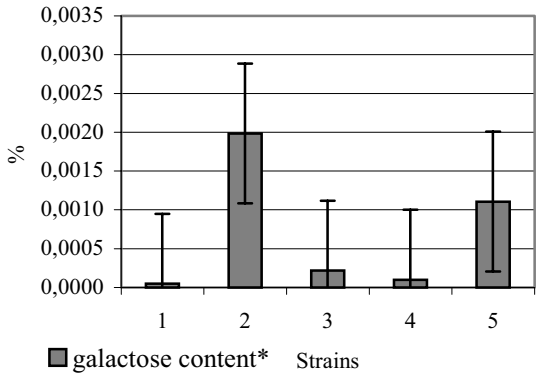


1 *K. lactis* NCAIM Y 00260 2 *K. marxianus* LAF 4
3. *K. marxianus* NCAIM Y 00933 4 *K. marxianus* NCAIM Y 00463
5 *K. marxianus* NCAIM Y 00697 * n = 3

Figure 2 Glucose levels (%) in the fermentation medium (means and 95% confidence intervals) at the end of the single-cell protein production process using various *Kluyveromyces* strains

Figure 2 indicates that *K. marxianus* NCAIM Y 00463 produced the significantly highest ($P < 0.05$) glucose level of all the strains tested in this study. The values reached by *K. marxianus* LAF 4 and *K. marxianus* NCAIM Y 00697 did not significantly differ ($P > 0.05$) from each other but were significantly higher ($P < 0.05$) than the results obtained for *K. lactis* NCAIM Y 00260 and *K. marxianus* NCAIM Y 00933. $LSD_{95\%} = 0.0009$.

3.4 Galactose Content of the Fermentation Medium

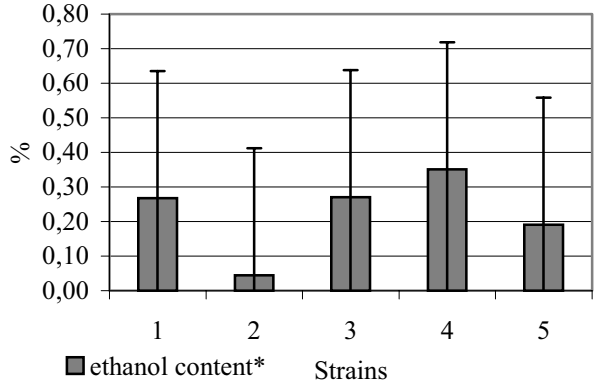


1 *K. lactis* NCAIM Y 00260 2 *K. marxianus* LAF 4
3 *K. marxianus* NCAIM Y 00933 4 *K. marxianus* NCAIM Y 00463
5 *K. marxianus* NCAIM Y 00697 * n = 3

Figure 3 Galactose levels (%) in the fermentation medium (means and 95% confidence intervals) at the end of the single-cell protein production process using various *Kluyveromyces* strains

It is shown in **Figure 3** that *K. marxianus* LAF 4 produced the significantly highest ($P < 0.05$) galactose level of all the strains tested in this study. The values reached by *K. marxianus* NCAIM Y 00933 and *K. marxianus* NCAIM Y 00697 did not significantly differ ($P > 0.05$) from each other. The results obtained for *K. lactis* NCAIM Y 00260 and *K. marxianus* NCAIM Y 00463 proved to be significantly lower ($P < 0.05$) than those obtained for *K. marxianus* LAF 4 or *K. marxianus* NCAIM Y 00697. $LSD_{95\%} = 0.001$.

3.5 Ethanol Content of the Fermentation Medium

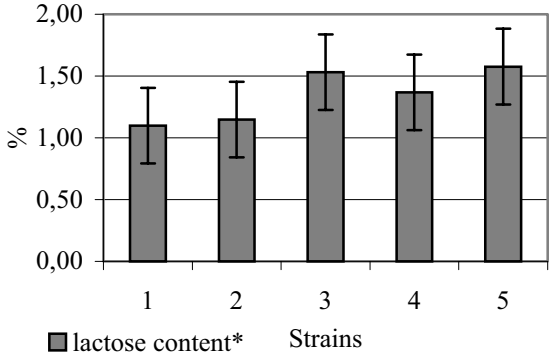


1 *K. lactis* NCAIM Y 00260 2 *K. marxianus* LAF 4
3 *K. marxianus* NCAIM Y 00933 4 *K. marxianus* NCAIM Y 00463
5 *K. marxianus* NCAIM Y 00697 * n = 3

Figure 4 Maximum ethanol levels (%) in the fermentation medium (means and 95% confidence intervals) produced by various *Kluyveromyces* strains

No significant differences ($P > 0.05$) were found between the various *Kluyveromyces* strains tested in terms of maximum ethanol levels measured in the fermentation medium (**Figure 4**).

3.6 Lactose Content of the Fermentation Medium



1 *K. lactis* NCAIM Y 00260 2 *K. marxianus* LAF 4
3 *K. marxianus* NCAIM Y 00933 4 *K. marxianus* NCAIM Y 00463
5 *K. marxianus* NCAIM Y 00697 * n = 3

Figure 5 Lactose levels (%) in the fermentation medium (means and 95% confidence intervals) at the end of the single-cell protein production process using various *Kluyveromyces* strains

The final lactose levels produced by *K. lactis* NCAIM Y 00260 and *K. marxianus* LAF 4 were significantly lower ($P < 0.05$) than those reached by *K. marxianus* NCAIM Y 00933 or *K. marxianus* NCAIM Y 00697, but did not significantly differ ($P > 0.05$) from the value obtained for *K. marxianus* NCAIM Y 00463 (**Figure 5**). $LSD_{95\%} = 0.433$.

Our results show that the screened strains differed in their β-galactosidase (EC 3.3.2.23) activity. *Kluyveromyces lactis* NCAIM Y 00260 and *K. marxianus* LAF 4 showed the highest lactase activity because their growth media had the lowest lactose concentrations by the end of the fermentation process. The medium used to grow *K. lactis* NCAIM Y 00260 also had decreased final galactose levels as compared to the other strains, and in terms of glucose content only *K. marxianus* NCAIM Y 00933 produced a value lower than that obtained for *K. lactis* NCAIM Y 00260. All these findings, together with the fact that *K. lactis* NCAIM Y 00260 reached the highest μ_{max} value (0.217 1/h), clearly indicate that this strain proved to be superior to all the other *Kluyveromyces* strains tested in terms of suitability for use in SCP production.

Although no significant differences ($P > 0.05$) were found between the strains tested in their maximum ethanol production, *K. marxianus* LAF 4 was less sensitive to oxygen limitation than the other strains, which all showed a slight diauxic growth.

3.7 Further Selection of Strains Based on Their Production Performance

Based on their μ_{max} values, two strains (i.e., *K. lactis* NCAIM Y 00260 and *K. marxianus* LAF 4) were selected for further SCP production trials. Temperature, pH, and agitation were set at 30°C, 4.5, and 300 rpm, respectively, and airflow rate was at 0.5, 1.0, 1.5, or 2.0 VVM during the experiments. Both data reported in the scientific literature and the possibility of later commercial applications were taken into consideration when the above operation parameters were determined.

3.7.1 Maximum total solids concentration (x_{max})

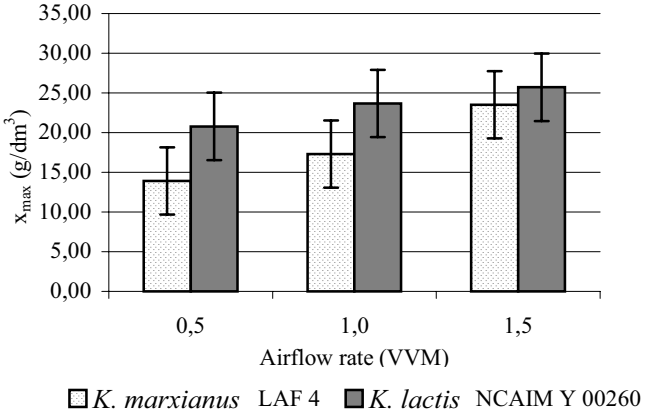


Figure 6 Maximum total solids levels (x_{max}) reached by the selected strains (means and 95% confidence intervals)

The maximum total solids values reached by both *K. lactis* NCAIM Y 00260 and *K. marxianus* LAF 4 at the airflow rate of 1.5 VVM were significantly higher ($P < 0.05$) than the corresponding results obtained at lower aeration levels (**Figure 6**). The x_{max} of *K. lactis* NCAIM Y 00260 at 1.0 VVM was the only exception to this rule. $LSD_{95\%} = 4.23$.

At the airflow rates of 0.5 VVM and 1.0 VVM, significant differences ($P < 0.05$) were found between the two strains in terms of their x_{max} values. $LSD_{95\%} = 3.45$.

3.7.2 Production rate (dx/dt)

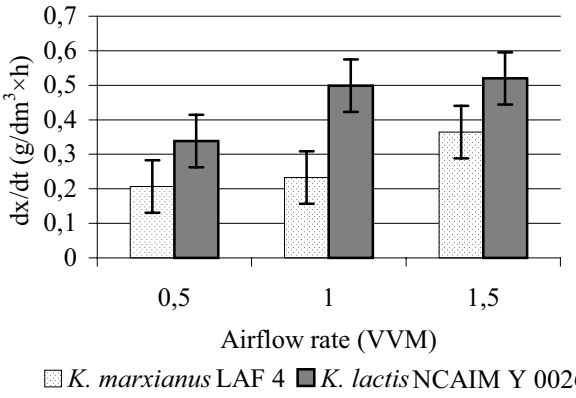


Figure 7 Production rates (dx/dt) of the selected strains (means and 95% confidence intervals)

The production rates of *K. lactis* NCAIM Y 00260 were significantly higher ($P < 0.05$) at 1.0 VVM and 1.5 VVM than at 0.5 VVM, whereas the dx/dt values obtained for *K. marxianus* LAF 4 were significantly higher ($P < 0.05$) at 1.5 VVM than at lower airflow rates (**Figure 7**). $LSD_{95\%} = 0.075$.

Kluyveromyces lactis NCAIM Y 00260 had significantly higher ($P < 0.05$) production rates at all three aeration levels than did *K. marxianus* LAF 4. $LSD_{95\%} = 0.062$.

3.7.3 Biomass yield ($Y_{x/S}$)

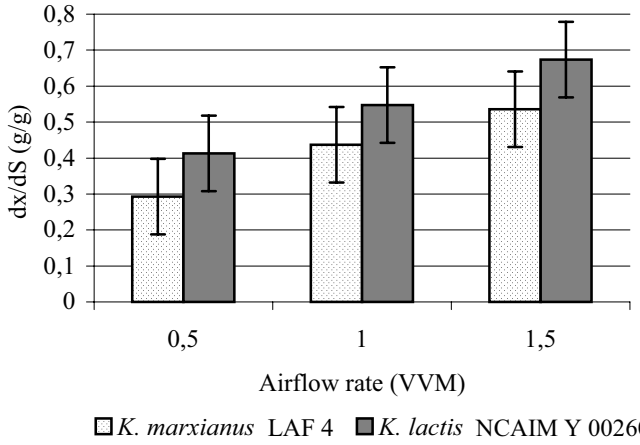


Figure 8 Biomass yield ($Y_{x/S}$) of the selected strains (means and 95% confidence intervals)

Figure 8 illustrates that biomass yield increased with increasing airflow rate. There were significant differences ($P < 0.05$) between the means of *K. lactis* NCAIM Y 00260 at all three aeration levels. As for *K. marxianus* LAF 4, the measured $Y_{x/S}$ values were significantly higher ($P < 0.05$) at 1.0 VVM and 1.5 VVM than at 0.5 VVM. $LSD_{95\%} = 0.105$.

Kluyveromyces lactis NCAIM Y 00260 produced significantly higher ($P < 0.05$) biomass yields at all three aeration levels than did *K. marxianus* LAF 4. $LSD_{95\%} = 0.068$.

3.8 Oxygen Demand of the *Kluyveromyces* Strains Tested

It is concluded from the results that the fermentation processes, i.e., the oxygen uptake rates by the yeast strains, were dependent on the oxygen concentration of the medium, therefore respiration rates (dc/dt), liquid-phase

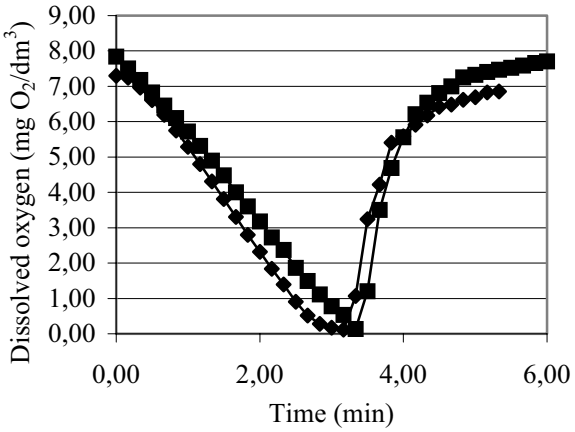
oxygen absorption coefficients (K_La), and saturation oxygen concentrations (C*) were determined for both strains at 1.5 VVM. For this, the following equation, which describes the respiration of microorganisms, was used:

$$dC/dt = K_La\times(C^*-C)-xQ \qquad (kg\ O_2/m^3\times h) \qquad [1],$$

where:
a: mass transfer surface per volume (cm^{-1})
K_La: liquid-phase oxygen absorption coefficient (h^{-1})
C*: saturation oxygen concentration (mg/dm^3)
C: measured dissolved oxygen concentration (mg/dm^3)
x: cell mass (g)
Q: specific respiration rate (h^{-1}).

3.8.1 Dynamic determination of K_La

Dissolved oxygen concentration (C) changes characteristically during fermentation. If this parameter is plotted against time, C* and K_La can be determined (**Figure 9**).



■ Dissolved oxygen levels* in the case of *K. marxianus* LAF 4
♦ Dissolved oxygen levels* in the case of *K. lactis* NCAIM Y 00260
* n = 3

Figure 9 Dynamic determination of K_La

Because dC/dt=-xQ when aeration is stopped, and x and Q are practically constant, the decreasing phase of the curve is a straight line. Its slope is the respiration rate. The respiration rates of *K. lactis* NCAIM Y 00260 and

K. marxianus LAF 4 in the decreasing phase of the curve (dC/dt) were found to be 2.85 mg $O_2/dm^3 \times min$ and 2.40 mg $O_2/dm^3 \times min$, respectively.

The K_La results calculated from the equation of the line fitted to the values obtained by plotting the linearized form of equation [1] were 227 h^{-1} and 187 h^{-1} for *K. lactis* NCAIM Y 00260 and *K. marxianus* LAF 4, respectively. C*, which is obtained by putting the dC/dt values in the equation of the line, was 6.63 mg/dm^3 in the case of *K. lactis* NCAIM Y 00260 and 7.5 mg/dm^3 for *K. marxianus* LAF 4.

4 NEW SCIENTIFIC FINDINGS

1. The fermentation parameters providing optimum conditions for *K. lactis* NCAIM Y 00260, *K. marxianus* LAF 4, *K. marxianus* NCAIM Y 00933, *K. marxianus* NCAIM Y 00463, and *K. marxianus* NCAIM Y 00697 to reach maximum viable cell counts and maximum specific growth rates during single-cell protein production were determined as follows: temperature of 30°C, pH of 4.5, agitation of 300 rpm, and airflow rate of 2.0 VVM. The glucose, galactose and ethanol levels produced under these optimum conditions in the fermentation media were measured, and the efficiency of lactose conversion was also quantified.

2. The maximum total solids concentration, biomass yield, and respiration rate values of *K. lactis* NCAIM Y 00260 and *K. marxianus* LAF 4 were determined during batch production of single-cell protein from sweet whey under the following operation conditions: temperature of 30°C, pH of 4.5, agitation of 300 rpm, and airflow rates of 0.5, 1.0, and 1.5 VVM.

3. Of all the *Kluyveromyces* strains tested, *K. lactis* NCAIM Y.00260 proved to be the strain most suitable for use in whey-based single-cell protein production.

5 SCIENTIFIC PUBLICATIONS AND PRESENTATIONS ON THE TOPIC OF THE Ph.D. DISSERTATION

Oral Presentations Given at Scientific Conferences and Symposia

In Hungarian

Kovács, P., Szigeti, J., **Ásványi, B.** (2003): Savó alapú egysejtfehérje előállítása. OMFB Szakmai beszámoló. Budapest.

Abstracts of Scientific Presentations

In Hungarian

Ásványi, B., Szigeti, J. (2003): Egysejtfehérje előállítására alkalmas élesztők szaporodásának összehasonlítása. MTA ÉKB – KÉKI – MÉTE 312. Tudományos Kollokvium előadásainak rövid kivonata. Budapest, 2003. szeptember. 284, 6.

Ásványi, B., Szigeti, J. (2002) Tejipari melléktermékek fehérje- és vitamintartalmának dúsítása (Enrichment of protein and vitamin contents in dairy by-products). *XXIX. Óvári Tudományos Napok "Agrártermelés – Életminőség"*. Az előadások és poszterek összefoglaló anyaga, Élelmiszer-tudományi Szekció, Mosonmagyaróvár, 100.

In English

Ásványi, B., Bugyi, G., Daróczi, L., Kovács, R., Szigeti, J., Varga, L. (2003) Growth of yeast strains during batch production of single-cell protein from cheese whey. *1st FEMS Congress of European Microbiologists*. Abstract Book, Ljubljana, 103–104.

Ásványi, B., Kovács, R., Szigeti, J., Varga, L., Kovács, P. (2003) Selection of *Kluyveromyces* strains for batch production of single-cell protein from cheese whey. *23rd International Specialised Symposium on Yeasts "Interaction Between Yeasts and Other Organisms"*. Book of Abstracts, Budapest, 156.

Kovács, R., Vecseri-Hegyes, B., **Ásványi, B.**, Varga, L., Szigeti, J., Daróczi, L., Bugyi, G. (2003) Manufacture of honey beer with *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*. *1st FEMS Congress of European Microbiologists*. Abstract Book, Ljubljana, 117–118.

Kovács, R., Vecseri-Hegyes, B., **Ásványi, B.**, Varga, L., Szigeti, J., Daróczi, L., Bugyi, G. (2003) Suitability of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* for use in honey beer production. *23rd International Specialised Symposium on Yeasts "Interaction Between Yeasts and Other Organisms"*. Book of Abstracts, Budapest, 188.

Ásványi, B., Szigeti, J., Varga, L. (2004) Suitability of *Kluyveromyces* spp. for use in single-cell protein production from sweet cheese whey. *American Dairy Science Association – American Society of Animal Science – Poultry Science Association 2004 Joint Annual Meeting*. Abstracts, St. Louis, Missouri: *Journal of Animal Science* **82** (Supplement 1) / *Journal of Dairy Science* **87** (Supplement 1) / *Poultry Science* **83** (Supplement 1) 384.

Papers Published in Proceedings

In Hungarian

Ásványi, B., Szigeti, J., Varga, L. (2003) Élesztőtörzsek szaporodásának összehasonlítása szakaszos egysejtfehérje-előállítási folyamatban (Comparing the growth of yeast strains during batch production of single-cell protein). *31. Műszaki Kémiai Napok*. Az előadások teljes terjedelemben megjelent anyagai, Veszprém, 295–299.

Ásványi, B., Szigeti, J., Varga, L. (2004) Savó alapú egysejtfehérje előállítás (Single-cell protein production from cheese whey). *XXX. Óvári Tudományos Napok "Agrártermelés – Harmóniában a Természettel"*. Az előadások és poszterek teljes terjedelemben megjelent anyagai, Élelmiszer-tudományi Szekció, Mosonmagyaróvár, Compact Disc. (Az előadások és poszterek összefoglaló anyaga, Élelmiszer-tudományi Szekció, Mosonmagyaróvár, 102.)

Peer-Reviewed Papers

In Hungarian

Ásványi, B., Szigeti, J., Varga, L. (2005) A savó, mint tejipari melléktermék élesztőgombákkal történő hasznosítása (Utilization of whey as a dairy byproduct by yeasts). *Acta Agronomica Óváriensis* **47** (2) (közlésre elfogadva).

Ásványi, B., Szigeti, J., Varga, L. (2005) *Kluyveromyces* törzsek összehasonlítása sajtsavó alapú szakaszos egysejtfehérje-előállítás során (Comparison of *Kluyveromyces* strains in terms of suitability for use in batch production of single-cell protein from cheese whey). *Acta Agronomica Óváriensis* **47** (2) (közlésre elfogadva).

In English

Ásványi, B., Reichart, O., Szigeti, J., Varga, L. (2006) Screening and selection of *Kluyveromyces* strains for use in batch production of single-cell protein from cheese whey. *Milchwissenschaft* **61** (accepted for publication).