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Yarrowia lipolytica Grown on Biofuel Waste as a Source of Single Cell Protein and Essential Amino Acids for Human Diet

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Abstract: Single Cell Protein (SCP) can be obtained from various microorganisms by growing them on a number of types of substrates, including industrial products waste. This work focuses on non-conventional yeast *Yarrowia lipolytica*, and its application in the process of production of valuable components like SCP and amino acids. It also analyses the possibility of using obtained biomass as a dietary supplement for humans. *Y. lipolytica* is nonpathogenic to humans and has been approved for use in several industrial processes as it is generally recognized as safe (GRAS). The results of our analyses suggest that the *Y. lipolytica* A-101 – a strain growing in biofuel production waste is a good candidate for a source of high-quality yeast protein (40-50% of dried biomass) and exogenous amino acids (phenylalanine 3.9 g, isoleucine 4.4 g, leucine 6.8 g, lysine 7.0 g, methionine 1.2 g, threonine 4.8 g, tryptophan 4.7 g, valine 5.3 g/100 g of protein). *Y. lipolytica* A-101 biomass has also very low nucleic acids concentration (below 1%) due to activation of endogenous nucleases in the final stage of the stationary phase. These enzymes reduce the amount of nucleic acids in this biomass from a high level to one acceptable for human consumption. Moreover, *Y. lipolytica* A-101 biomass is safe and digestible since the yeast cells get killed in the drying process and has no leavening powder with destroyed cell wall. *Y. lipolytica* A-101 can be used as a nutritional supplement in human diet when an increased intake of amino-acids and SCP is required.

Keywords: Biofuel waste, dried biomass, non-conventional protein source, *Yarrowia lipolytica*, single cell protein.

INTRODUCTION

Due to the rapidly increasing world human population and consequently the possibility of widespread deficiency of protein, since the early fifties, the world of science has been exploring new, alternative and unconventional ways of producing sources of protein [1]. This resulted in an alternative protein obtained from various microorganisms named "Single Cell Protein" (SCP), a protein biomass, bioprotein or microbial protein. SCP contains higher amount of protein than its cousins derived from plant and animal sources [2]. Bacteria, yeast, fungi and algae can produce protein biomass, which after drying is used as a supplement in human foods or animal feeds. Besides high protein content (in yeast and fungi it is about 50-55% of dry cell weight), SCP also contains fats, carbohydrates, nucleic acids, vitamins and minerals. SCP is rich in many essential amino acids like lysine and methionine which are limited in most plant and animal foods [1, 3, 4]. SCP can also be used as a low cost addition to the main diet instead of sources known to be relatively expensive such as soybean and fish [4].

Among microorganisms, yeasts are widely used as source of SCP and the ones suitable for consumption are called nutritional yeasts. Their features are a wide amino acid spectrum and high protein-carbohydrate ratio, which makes them particularly attractive as a high-nutrient feed substitute [5]. Moreover, nutritional yeasts are environmental friendly. They are able to grow on waste, therefore reducing the environmental pollution and aiding waste recycling [3].

The most popular of yeast species are *Saccharomyces* and the oily yeasts genera such as *Candida* and *Yarrowia* [1, 6]. Most microorganisms, especially yeasts, utilize inexpensive feedstock and waste to produce biomass, protein concentrate and/or

amino acids. Conventional substrates such as starch, molasses, fruit and vegetables wastes have been used for SCP production, as well as unconventional ones such as petroleum by-products, natural gas, ethanol, methanol, lignocellulosic biomass and animal-waste fats [1, 7-11]. The method of harvesting, drying and processing has an effect on the nutritive value of the final product [12]. The organisms like yeast grow faster than plant or animal sources and produce large quantities of bioprotein from relatively small area of land and within a short time [3]. For example, *Candida* spp., which use brewery (distillery sludge) or food production waste (rice bran) as a substrate produce about 30% and 33% SCP in their biomass, respectively [13, 14]. Nevertheless, *Candida spp*. grown in *n*-alkanes could produce 65% crude protein. In turn, *C. utilis* utilizing ethanol, sulphite waste liquor, contains 50-55% protein in biomass [3, 15]. Consecutively, *Yarrowia lipolytica* is oleaginous yeast that grows on a variety of hydrophobic substrates, especially different fractions of petroleum, as well as streams from different industries [16-19]. *Y. lipolytica* is nonpathogenic to humans. The natural occurrence of this yeast in food, particularly in cheese and meat, is an argument supporting its safety claim and several production processes using *Y. lipolytica* have been granted "Generally Regarded as Safe (GRAS)" status by the US FDA [18].

The use of SCP as an additional nutrition supplement can support a solution to the problem of food scarcity in rapidly growing population areas, especially in developing countries like India [1]. The data published by the Food and Agriculture Organization (FAO) suggests that 25% of the world population has protein deficiency [4]. Moreover, SCP can be regarded as an alternative source of protein for people who avoid eating meat, especially vegans and vegetarians [20]. Currently, there are several food spreads commercially available made of yeast extract (especially *Saccharomyces cerevisiae*), dietary supplements containing brewer's yeast and a meat substitute product delivered from *Fusarium venenatum* fungus. These products are sold in a number of countries around the world. However, the use of SCP as food ingredient is still in development. Obtaining SCP from microorganisms could be improved by developing genetic engineering procedures for mass production of these proteins [3].

Admittedly, for SCP to be commercially successful in the future, food technology problems have to be solved first, but these refinements have been achieved in manufacturing processes of many similar products already available [1]. However, SCP main advantages which are high yields, very good nutritional level and documented safety record make strong argument for its inclusion as diet supplement for both human and livestock [3].

The presented study was carried out to assess the potential of biofuel waste in the production of *Yarrowia lipolytica*, and its dried biomass serving as a source of SCP and amino acids for human in addition to animal consumption.

MATERIALS AND METHODS

Microbial Strains

We used yeast *Yarrowia lipolytica* A-101 obtained from Skotan S.A. Poland. In some experiments the reference yeast strain *Yarrowia lipolytica* ATCC 9773 obtained from LGC Standards was included.

Production and harvesting of SCP

Submerged fermentations were carried out on laboratory and pilot plant scale. On the laboratory scale, cultures of both *Y. lipolytica* strains were cultivated in *Erlenmeyer* flasks (150 ml) with two trial media: YPD medium (Difco) and SK medium being waste from biofuel production (mix of vegetable oils, degumming and glycerol fractions formed during biofuel production). SK medium was provided by Skotan S.A. (Poland). In all the media, pH was adjusted to 4.0, 5.0, 6.0 or 7.0 using 1N HCl and/or 1N NaOH, respectively. Each medium was being sterilized at 121°C for 15 minutes. Inoculum for all cultivations was prepared by transferring 2-day old culture with initial OD_{650} around 0.15 followed by cultivation in shaking flask or biofermentor as standard production of industry. On the laboratory scale, fermentation was carried out at 20, 25 or 30°C at 200 rpm in an incubator shaker followed by determination of biomass and other parameters after 12 or 18 h intervals. On the pilot plant scale, cultures of *Y. lipolytica* A-101 were cultivated in biofermentors (10 L or 100 L) in SK medium at temperature of 30°C and pH 5.0 with mechanical agitation and 40% oxygenation. In biofermentors, the cultures were conducted for 12 hours. After fermentation, biomass was separated from culture medium by centrifugation at 8000 x g for 15 minutes in order to pellet down the yeast cells and washed three times with sterile water. Biomass obtained from biofermentor was transferred into a tumble dryer and dried at 165-175°C for one hour obtaining dried biomass as Yarrowia powder.

Chemical analysis

Proximate analysis of the yeasts biomass: crude protein and water contents were determined by AOAC methods [21]. The amino acids analysis was performed according to the method of Moore and Stein [22].

STATISTICAL ANALYSIS AND MANAGEMENT DATA

All data are expressed as mean ± SD (standard deviation) of three independent experiments. The differences between concentrations of total protein and nitrogen for *Y. lipolytica* A-101 cultivated on SK medium at pH 5.0 and other conditions were

determined by two sided student's *t*-test by using Statistica software 12.0 version. A *P* value <0.05 was considered statistically significant.

Nucleic acids examination

Nucleic acids were isolated from yeast biomass by hydrolization using 2 N H_2SO_4. The RNA and DNA content in the samples was determined by the orcinol method or diphenylamine method, respectively [23].

RESULTS AND DISCUSSIONS

Single Cell Protein

We compared the feasibility of obtaining SCP from standard laboratory YPD medium and industry SK medium (waste of biofuel production) using *Y. lipolytica* A-101 to produce biomass in comparison with reference strain *Y. lipolytica* ATCC 9773. In our experiments on the laboratory scale (Fig. 1), considerable growth of both strains was observed in YPD medium, irrespective of pH. Similar data were obtained in case of *Y. lipolytica* A-101 grown in SK medium. However, *Y. lipolytica* ATCC 9773 did not grow in low pH (4.0 or 5.0) in SK medium. The results of protein production revealed that temperature of 30°C and pH 6.0 (72 g/kg wet biomass) was better for cultivation of the ATCC 9773 strain in SK medium than lower temperature (20°C or 25°C) with the same pH value. Using SK medium and the A-101 strain, the highest protein contents were obtained under the following specific conditions: temperature of 30°C and pH 4.0 (110 g/kg wet biomass). In this case, the result is almost the same, non significant difference as one obtained during cultivation of this strain in YPD medium (116 g/kg wet biomass). However, sufficient protein contents were also obtained at the temperature of 30°C and pH 5.0 (85 g/kg wet biomass).

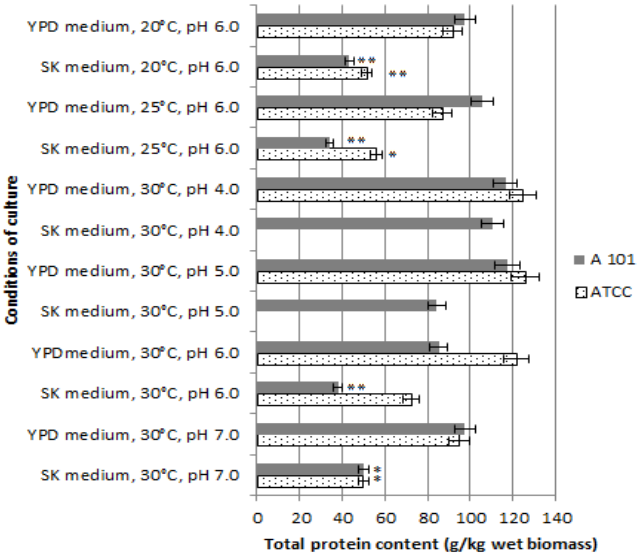

Fig.1. Total protein content in wet biomass of *Y.lipolytica* strains in various culture conditions and substrates (YPD medium and SK medium i.e. waste of biofuel production) on laboratory scale.

A-101 - *Y.lipolytica* A-101; ATCC - *Y.lipolytica* ATCC 9793.
**P*<0.05 and ***P*<0.01 indicate significant difference compared with reference cultivation (pH 5.0)

Results of nitrogen and protein percentage contents in wet biomass of *Y. lipolytica* A-101 grown in SK medium on laboratory scale are presented in Table 1. The most promising results were obtained under the conditions of the temperature of 30°C and pH 4.0 or 5.0. In this environment the total nitrogen and protein contents in wet biomass were recorded as 10.89% or 8.28% and 1.74% or 1.35%, respectively. However, these differences were not statistically significant. Much lower nitrogen and protein contents were obtained in pH 6.0 or pH 7.0 that is 3.64% or 4.84% for nitrogen and 0.58% or 0.77% for protein, respectively, as compared to the values obtained in pH 5.0. These differences were statistically significant. It confirms that *Y. lipolytica* A-101 showed good ability to produce high yields of protein cultivated in SK medium, i.e. waste of biofuel production at lower pH. In a similar manner, *Y. lipolytica* ACA-DC 50109, growing on a solid industrial animal-waste fats derived from beef tallow, produced single cell protein [24].

Table-1: Total nitrogen and protein content in biomass of *Yarrowia lipolytica*A-101 strain growing under various culture conditions in SK medium (biofuel production waste) on laboratory scale. Conditions of cultivation: 150 ml, 30°C, 200 rpm, 18 h. * *P*<0.05 and ** *P*<0.01 indicate significant difference compared with reference cultivation (pH 5.0)

pH	Total nitrogen content [%] of wet biomass, mean ± SD	Total protein content [%] of wet biomass, mean ± SD
4.0	10.89 ± 1.40	1.74 ±0.22
5.0	**8.28 ± 1.11**	**1.32 ± 0.18**
6.0	3.64 ± 1.25 **	0.58 ± 0.20 **
7.0	4.84 ± 1.82 *	0.77 ± 0.29 *

SD – standard deviation

After the optimization of culture conditions on the laboratory scale we carried out biofermentor's growth on the pilot plant scale. The protein contents of dried yeast biomass obtained from seven batches of independent biofermentor standard production were also determined (Table 2). In the biofermentor, optimized conditions for the SCP production from *Y. lipolytica* A-101 in waste of biofuel production (SK medium) were estimated to be: temperature of 30°C and pH 5.0. In case of standard production in biofermentor it was recorded that the average yeast protein contents were consistently 45.6% of dry weight, in the range 41.9%-49.3%. Mean nitrogen content was 7.1%, in the range from 6.7% to 7.9%. Our findings support research made by Zhao *et al*. [11] showing that cultivation of a recombinant strain of *Y. lipolytica* SWJ-1b on different media allows obtaining both high protein content and the antimicrobial peptide (AMP). Biomass of this strain contained from 45.3% to 48.9% of crude protein per 100 g of dry cell weight [11].

Table-2: The composition of *Yarrowia lipolytica* A-101 dried biomass (Yarrowia powder) obtained after culturing in SK medium (biofuel production waste) on pilot plant scale. Conditions of cultivation: 100 L, 30°C, pH 5.0, 40% oxidation, 12 h

Batch number[a]	Average composition (% of dry weight) ± SD			
	Water	Nitrogen (N)	Crude Protein	Nucleic acids
1.	5.7 ± 0.21	7.9 ± 0,10	49.3 ± 0.63	0.7 ± 0.14
2.	4.3 ± 0.18	6.7 ± 0.27	41.9 ± 1.67	0.8 ± 0.07
3.	4.0 ± 0.16	6.8 ± 0.17	42.6 ± 1.07	1.0 ± 0.14
4.	5.9 ± 0.22	7.0 ± 0.11	43.7 ± 0.69	0.7 ± 0.00
5.	4.1 ± 1.21	7.1 ± 1.24	44.8 ± 1.24	not tested
6.	3.1 ± 1.12	6.7 ± 0.71	42.0 ± 0.71	1.0 ± 0.07
7.	3.7 ± 0,90	6.7 ± 0,18	41.9 ± 1,10	0.8 ± 0.14
Mean	4.2 ± 0.46	7.1 ± 0,15	45.6 ± 0,87	0.8 ± 0.09

SD – standard deviation

[a]Each of the batches were obtained from different and independent cultivations

Despite having nutritional value, a yeast protein should also have desirable functional properties for its incorporation in food. Although, the cell wall of the yeasts may be non-digestible and there may be unacceptable colour and flavours, this can be greatly improved by killing the organisms before consumption [3] The digestibility of yeast can also be greatly increased by drying it at high temperature under certain conditions [25]. Drying is one of the methods most commonly used in food preservation as it allows to obtain food products with a longer shelf life. It is a technique that has increased in popularity in the last century as the demand for food stability has risen due to the increase in world population. This higher food demand has influenced quite significantly the relevance of preservation by drying [26]. In this method, the moisture content of food powders is usually between 2 and 8%. At this level, powders are stable with an average shelf life of 12 – 24 months. Drying is implemented before or after grinding operations and plays a major role in the texturing and stabilization of food materials [27]. In our experiments, results obtained for water contents of each of the batches of dried biomass studied were in the range between 3.1% and 5.9% (Table 2).

Nucleic acids

The high amount of nucleic acid (DNA and RNA) in SCP could be removed or reduced from about 7% to 1%, which would then be considered to be within the acceptable level [3, 25, 28, 29]. The consumption of protein biomass with high nucleic acid concentration (6-10%) is undesirable as it elevates uric acid levels in blood, inducing health disorders such as gout and kidney stone [25, 30, 31].

It was shown on the laboratory scale (Figure 2) that *Y. lipolytica* A-101 presented the high nucleic acids content during growing from initial lag phase (6.4%) to the end of log phase (5.8%). However, in the stationary phase we observed a dramatic reduction of nucleic acids concentration (below 1.0%), especially at the end of this stage (0.4%). Additionally, the concentration of nucleic acids in the dried biomass obtained from the pilot plant standard production (Table 2) was low in all tested batches.

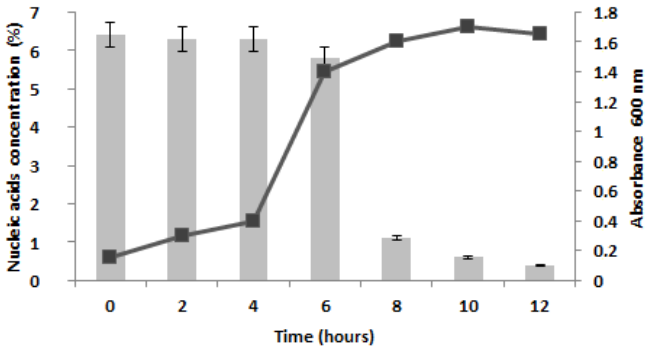

Fig.2. Nucleic acid concentration during the growth phases of *Y. lipolytica* A-101 culturing in SK medium (biofuel production waste) on laboratory scale.

Amino acids

In our experiments, we estimated the ability of *Y. lipolytica* to produce amino acids in biomass under optimal conditions for SCP production: the industry SK medium (waste of biofuel production), temperature of 30°C and pH 5.0. Results obtained from dried biomass of *Y. lipolytica* A-101 cultivated in standard bioreactor condition in biofuel production waste are presented in Table 3. We compared results of essential amino acid contents with FAO recommendations for human nourishment and amino acid composition of *Candida utilis* [3] (Table 3). Obtained levels of these amino acid contents from *Y. lipolytica* A-101 cover completely FAO requirements for isoleucine, leucine, lysine, phenylalanine, threonine and valine. Yarrowia SCP is rich in isoleucine, leucine, phenylalanine, threonine, valine and alanine, arginine, asparagine, glutamine, glycine, histidine and especially tyrosine, the content of which is comparatively higher than that of *C. utilis*. Our results indicate that *Y. lipolytica* is poor in amino acids containing sulphur. Levels of methionine and cysteine cover about 50% of the nutrient reference values for these amino acids.

Table-3: Amino acid composition of *Yarrowia lipolytica* A-101 powder obtained from yeast biomass growing in SK medium (biofuel production waste) on pilot plant scale in comparison with *Candida utilis* and FAO requirements. Conditions of cultivation: 100 L, 40% oxidation, 30°C, pH 5.0, 12 h.

	Amino acid contents			
Amino-acid	*Yarrowia lipolytica* (g/kg dried biomass), mean±SD	*Yarrowia lipolytica* (mg/g protein), mean±SD	*Candida utilis*## (mg/g protein)	FAO## (mg/g protein)
Aspartic acid + asparagine	29.6 ± 0.25	88 ± 5.09	66.5	-
Threonine#	16.1 ± 0.50	48 ± 0.21	34	40
Serine	14.7 ± 0.55	44 ± 0.28	36	-
Glutamic acid + glutamine	40.6 ± 3.55	120 ± 9.48	90.5	-
Proline	14.3 ± 0.25	42 ± 2.97	-	-
Glycine	15.5 ± 0.50	46 ± 0.07	28	-
Alanine	27.2 ± 3.35	80 ± 10.32	46	-
Valine#	18.1 ± 0.25	53 ± 1.41	40,5	42
Isoleucine#	15.0 ± 0.05	44 ± 2.33	32	42
Leucine#	23.1 ± 0.50	68 ± 5.30	44	48
Tyrosine	37.5 ± 10.75	110 ± 40.09	26	-
Phenylalanine#	13.4 ± 0.25	40 ± 0.78	30	28
Histidine	8.9 ± 0.55	26 ± 0.99	16	-
Lysine#	23.6 ± 0.70	70 ± 6.22	76	42
Arginine	16.3 ± 0.70	48 ± 5.16	38	-
Cysteine	3.7 ± 1.90	11 ± 8.49	24	20
Methionine#	4.0 ± 1.00	12 ± 4.74	15,5	22
Tryptophan#	15.9 ± 3.85	47 ± 18.31	-	-

#Exogenous amino-acids; ##(Adedayo et. al., 2011); SD - standard deviation

CONCLUSION

SCP of *Yarrowia lipolytica* shows very great potential as a nutrition supplement for humans. Dried biomass of *Y. lipolytica* has 40-50% yeast protein, as well as a good balance of amino acids, especially indispensable amino acids. Yarrowia powder contains very low, safe for human consumption, level of nucleic acids concentration (below 1%). Additionally, the water content of Yarrowia powder is below 6 % and the low moisture content of this yeast's dried biomass makes it suitable for use as an ingredient in preparation of dietary supplement and other human food or animal feed. Since Yarrowia powder is obtained by a drying process at very high temperature which kills the yeast cells and destroys the cell wall, it results in its improved digestibility.

Yarrowia lipolytica is characterized by a high rate of multiplication, and can be grown on a variety of carbon sources like biofuel production waste, which helps recycling and reduces the carbon footprint. Yeast obtained in this way can then be easily separated by centrifugation due to their large cells. *Y. lipolytica* is nontoxic by-product in the production of biomass, yeast protein and amino acids and its growth is independent of seasonal and climatic conditions. However, the biggest advantage of nutritional yeast is its high protein content, appealing particularly for people who avoid eating meat, especially vegans and vegetarians, as well as people who live in poor regions and throughout the world with limited food sources. 100 gram of Yarrowia powder contains approximately 50 grams of protein. This is almost 100 percent of an adult's recommended daily intake (50 grams) [32]. Moreover, Yarrowia powder contains all indispensable and conditionally essential amino acids, making it a "complete" protein.

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