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A STUDY ON THE PRODUCTION OF POLY-BETA-HYDROXYBUTYRATE BY SOME EUKARYOTIC MICROORGANISMS

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Özet

Bu çalışmada, kambuçya çayından izole edilen 15 maya izolatı, identifikasyon sonucunda *Saccharomyces cerevisiae*, *Candida krusei*, *Kloeckera apiculata* ve *Kluyveromyces africanus* olarak teşhis edilmişlerdir. Ayrıca bu çalışma için A.Ü.Z.F.'den 22 adet farklı maya suşu temin edilmiştir. Araştırmada kullanılan mayaların hücre kuru ağırlığına göre yüzde PHB verimleri, kambuçya çayından izole edilen mayalarda %0,50-16,67 değerleri arasında bulunurken, A.Ü.Z.F.'den temin edilen maya türlerinde %0,25-21,74 değerleri arasında olduğu belirlenmiştir. Çalışmada ayrıca farklı karbon ve azot kaynaklarının, PHB üretimi yüksek olan *Rhodotorula glutinis* var. *glutinis* 60 ve *Saccharomyces diastaticus* 27'nin PHB üretimi üzerine etkisine bakılmıştır. Farklı azot kaynaklarının *Rhodotorula glutinis* var. *glutinis* 60'da PHB üretimini arttırmadığı belirlenirken, karbon kaynaklarından mannitollü ortamda PHB veriminin (%21,95) kontrole göre daha yüksek olduğu görülmüştür. *Saccharomyces diastaticus* 27 suşunun farklı karbon kaynaklarının PHB verimini artırıcı bir etkisi olmadığı belirlenirken, azot kaynaklarından DL-triptofan içeren ortamda geliştirildiğinde PHB veriminin %25'e yükseldiği gözlenmiştir.
Anahtar Kelimeler: Kambuçya çayı, Maya, İzolasyon, İdentifikasyon, Poli-β-hidroksibütirat (PHB)

Abstract

In this research, 15 yeast strains were isolated from kombucha tea. As a result of the identification tests, these strains were identified *Saccharomyces cerevisiae*, *Candida krusei, Kloeckera apiculata* and *Kluyveromyces africanus*. Also, 22 different strains of yeast were taken from A.U.Z.F for this research. While the PHB percent according to cell dry weight of yeast strains isolated from Kombucha tea were found to be between 0.50-16.67%, the PHB percent according to cell dry weight of yeast strains taken at A.U.Z.F. were determined between 0.25-21.74%. Besides, the effects of different carbon and nitrogen sources on the accumulated PHB were examined in *Rhodotorula glutinis* var. *glutinis* 60 and *Saccharomyces diastaticus* 27 strains that had high PHB production. Different nitrogen sources were determined not to increase amount of PHB percent in *Rhodotorula glutinis* var *glutinis* 60. On the other hand, when the strain was grown in mannitol medium as carbon source, the PHB percent production was observed higher than control and the PHB percent production increased to 21.95%. While the strain *Saccharomyces diastaticus* 27 was determined not to have any effect to increase the PHB percent production of different carbon sources, when it was grown in DL-tryptophan medium from nitrogen sources the PHB percent production was observed to increase to 25%.
Key Words: Kombucha tea,Yeast, Isolation, Identification, Poly-β-hydroxybutyrate (PHB)

Introduction

The tea fungus, which also known as Kombucha tea, is a mixed culture of acetic acid bacteria and yeasts. Kombucha is a popular beverage among many traditional fermented foods across the world. It originated in northeast China (Manchuria) and later spread to Russia and the rest of the world (1, 2, 3, 4). The consumption of fermented tea was firstly practiced in 220 B.C. in Manchuria. It, then, spread to Russia were a wide litterature on teakwas is avaliable (2). Presently, its consumption is popular in the world, this popularity is mainly due to its refreshing power because of its low ethanol content (2). The researchers reported that the components of kombucha tea beverage are as follows: ethanol, gluconic, L-lactic, acetic acid, tartaric, succinic, malic, citric, oxalic and pyruvic acid, purines, pigments, lipids and proteins, enzymes, vitamins of B group and vitamin C, antibiotically active substances, usnic acid, as well as insufficiently known products of yeasts and bacteria metabolism (2, 5, 6, 7, 8, 9). Greenwalt et al. (2000) reported that kombucha proved itself effective at easing and promoting digestion, and headaches, hemorrhoids, atherosclerosis, metabolic disorders, gout, arthritis, diabetes, sluggishness of the bowels, fatigue, stress, old age and cancer were to be cured by regular consumption of kombucha. Sterile

containers and utensils must be used during kombucha preparation in order to prevent contamination from airborne molds or pathogenic organisms. The kombucha recipe may vary; it is commonly made by infusing black tea leaves into freshly boiled water, sweetened with sucrose, for about 10 min. After removing the tea leaves, the tea is allowed to cool to room temperature and the microbial mat/colony from a previous batch is added to sweetened tea with about 100 ml of kombucha from a previous fermentation. It is then covered with a clean cotton cloth and incubated at room temperature for about 7 to 10 days, the acidity may rise to levels potentially harmful to consume. Next, the microbial colony is removed and the kombucha beverage is ready (1, 8, 9). The exact microbiological composition depends on the source of inoculums of the tea fermentation. Numerous researchers reported that *Acetonobacter xylinum* oxidizing ethanol to acetic acid is the primary prokaryotic bacterium in tea. The yeast composition of tea is highly variable; but that, *Brettanomyces, Zygosaccharomyces, Saccharomyces, Torula, Candida, Mycoderma, Mycotorula, Pichia* occurred most frequently in kombucha tea (9). In addition, Greenwalt et al. (2000) reported that Roussin tested about 900 samples and never found the presence of the human pathogen *Candida albicans*.

Poly-3-hydroxybutyrate (PHB) is one of the polyhydroxyalkanaotes synthesized by bacteria as carbon and energy materials (10, 11, 12). Over the past trecade this material has received much attention as a biodegradable thermoplastic, although microbial production costs have limited its commercial exploitation. PHB synthesis is generally regarded as foreign to eukaryotic cells, although evidence exists that yeast and many other eukaryotic cells contain small amounts of low molecular mass PHBs which function as complexes with polyphosphate in membrane transport (13). Eukaryotic production of PHB through genetic engineering, especially in plants, is being pursued as a potentially inexpensive alternative to prokaryotic production (10, 11, 12, 13).

Our searching of literature showed that PHB synthesis is studied generally in prokaryotic cells, but the content of PHB in eukaryotic cells are not well documented. The purpose of this work was to obtain information about the PHB synthesis in eukaryotic cells. It is necessary to determine most suitable carbon and nitrogen sources to microorganism to decrease the production cost at minimum level and increase the production. In this study, for this reason the effect of different carbon and nitrogen sources on the yeast production PHB was examined.

Materials and Methods

Yeast Strains

The yeast strains used are listed in Table 1.

Table 1. The yeast strains used in study

Strains	**Source**
Saccharomyces cerevisiae S1	G.Ü.
Saccharomyces cerevisiae S2	G.Ü.
Saccharomyces cerevisiae S3	G.Ü.
Saccharomyces cerevisiae S4	G.Ü.
Saccharomyces cerevisiae Z1	G.Ü.
Saccharomyces cerevisiae Z2	G.Ü.
Saccharomyces cerevisiae Z4	G.Ü.
Saccharomyces cerevisiae Z5	G.Ü.
Candida krusei S6	G.Ü.
Candida krusei S7	G.Ü.
Candida krusei N1	G.Ü.
Candida krusei N2	G.Ü.
Kloeckera apiculata Z3	G.Ü.
Kloeckera apiculata N3	G.Ü.
Kluyvermyces africanus N4	G.Ü.
Saccharomyces cerevisiae KII 2	A. Ü. Z. F
Saccharomyces cerevisiae K119	A. Ü. Z. F
Saccharomyces cerevisiae 22	A. Ü. Z. F
Saccharomyces cerevisiae 26	A. Ü. Z. F
Saccharomyces cerevisiae 5	A. Ü. Z. F

Saccharomyces cerevisiae DHW 12	A. Ü. Z. F
Saccharomyces cerevisiae 6815	A. Ü. Z. F
Saccharomyces cerevisiae 32	A. Ü. Z. F
Saccharomyces cerevisiae 34	A. Ü. Z. F
Saccharomyces cerevisiae 43	A. Ü. Z. F
Saccharomyces bayanus 1A18	A. Ü. Z. F
Saccharomyces pasterianus 4	A. Ü. Z. F
Saccharomyces diastaticus 27	A. Ü. Z. F
Saccharomyces baillii var. *bailii* 46 (24 G)	A. Ü. Z. F
Schizosaccharomyces pombe 57 (24 A)	A. Ü. Z. F
Kloeckera apiculata 58	A. Ü. Z. F
Rhodotorula glutinis var. *glutinis* 60	A. Ü. Z. F
Candida membranaefaciens (No.1) 61	A. Ü. Z. F
Candida tropicalis Cappo (571) 64	A. Ü. Z. F
Candida lipolytica 66	A. Ü. Z. F
Hansenula anamola var. *anamola* IB (T) 69	A. Ü. Z. F
Kluyveromyces dobshankii 2A73	A. Ü. Z. F

Isolation and Identification

The yeast strains were isolated in YEPD medium (Yeast Extract Peptone Dextrose) by dilution, and the strains were incubated at 30^{o}C for 24 h (14). Each 100 ml of the YEPD Agar medium contained 2 g dextrose, 2 g peptone and 2 g yeast extract and 2 g agar. Standard biochemical tests were used for yeast identification. The colony morphology, the growth of different temperature (26^{o}C, 30^{o}C, 37^{o}C and 50^{o}C) were examined. For determination of carbon source utilization, arabinose, threhalose, xylose, raphinose, maltose, mellesitose, sorbitol, galactose, sellobiose, mellibiose, sucrose, glucose, lactose, mannitol, ramnose were used. Starch and gelatine hydrolise, nitrate reduction, urease test were determined (14, 15, 16).

Determination of PHB

Determination of the amount of PHB was performed chemically. The amount of PHB was determined on a spectrophotometer, wavelength 235 nm (17). The correlation between production of PHB and dry cell weight was determined by Spearman's test (18).

The effect of production of PHB in different carbon and nitrogen sources

After dextrose in YEPD medium broth was taken out, the ratio 2% mannitol, sucrose and arabinose were added to the medium as carbon sources. Peptone was taken out, and the ratio 1% L-cysteine, $(NH_4)_2SO_4$, glycine, DL-tryptophan and protease peptone were added as nitrogen sources. Carbon and nitrogen sources were sterilized by Millipore filter with a pore size of 0.45 μm.

Results and Discussion

In this research, 15 yeast strains were isolated from kombucha tea. As a result of the identification tests, these strains were identified *Saccharomyces cerevisiae*, *Candida krusei, Kloeckera apiculata* and *Kluyveromyces africanus*. The researchers found that the yeast strains in tea fungus are *Pichia* sp.,

Zygosaccharomyces kombuchaensis, *Z. rouxii*, *Z. pombe*, *Brettanomyces* sp., *S. cerevisiae*, *S. ludwigi*, *Candida* sp. and *Kloeckera apiculata* (2, 3, 4, 6). Chen and Liu (2000) reported that the bacteria isolated were identified as *Acetobacter aceti* and *Acet. pasteurianus*, while the yeasts were *S. cerevisiae*, *Zygosaccharomyces bailii* and *Brettanomyces bruxellensis* (19).

Table 2 shows the content of PHB in isolated yeast strains. The amount of PHB in these strains was 0.02-0.15 g/l, and the percentage of PHB in these cells was between 0.50 and 16.67% of dry cell weight. While the PHB productivity percentage in *Kloeckera apiculata* Z3 was the highest (16.67%), the lowest PHB productivity was found in *Saccharomyces cerevisiae* Z2 (0.50%).

Table 2. The PHB content of the yeast strain isolated from Kombucha tea

Yeast Strains	Dry Cell Weight (g/l)	[a]PHB (g/l)	[b]Yield of PHB (%)
Saccharomyces cerevisiae S1	6.14 ± 0.44	0.05 ± 0.02	0.81
Saccharomyces cerevisiae S2	1.04 ± 0.10	0.04 ± 0.01	3.85
Saccharomyces cerevisiae S3	1.025 ± 0.06	0.05 ± 0.02	4.88
Saccharomyces cerevisiae S4	4.70 ± 1.43	0.06 ± 0.01	1.28
Saccharomyces cerevisiae Z1	2.78 ± 0.23	0.05 ± 0.02	1.80
Saccharomyces cerevisiae Z2	3.98 ± 0.17	0.02 ± 0.01	0.50
Saccharomyces cerevisiae Z4	5.56 ± 0.22	0.06 ± 0.01	1.08
Saccharomyces cerevisiae Z5	5.62 ± 0.12	0.03 ± 0.00	0.53
Candida krusei S6	2.81 ± 0.21	0.07 ± 0.01	2.49
Candida krusei S7	3.11 ± 0.22	0.08 ± 0.01	2.57
Candida krusei N1	1.62 ± 0.30	0.15 ± 0.00	9.26
Candida krusei N2	1.64 ± 0.11	0.04 ± 0.00	2.44
Kloeckera apiculata Z3	0.24 ± 0.01	0.04 ± 0.00	16.67
Kloeckera apiculata N3	1.43 ± 0.33	0.14 ± 0.02	9.79
Kluyveromyces africanus N4	1.52 ± 0.05	0.10 ± 0.02	6.58

[a]Determined cell dry weight.
[b]According to cell dry weight.

The amount of PHB in the yeast strains obtained from A.U.Z.F. was found to be 0.03-0.43 g/l, the percentage of PHB in these cells was determined between 0.25 and 21.74% of dry cell weight. The maximum PHB yield in *Rhodotorula glutinis* var. *glutinis* 60 and *Saccharomyces diastaticus* 27 was found to be respectively of 21.74% and 19.73% (Table 3). The content of PHB of *Saccharomyces diastaticus* 27 was shown to be high. The relationship between the dry cell weight of yeast species and PHB production was tested and it was found that a significant relationship didn't exist between dry cell weight and PHB production.

Table 3. The content of PHB in different yeast strains

Strains	Dry Cell Weight (g/l)	[a]PHB (g/l)	[b]Yield of PHB (%)
Saccharomyces cerevisiae KII 2	7.86 ± 0.38	0.16 ± 0.13	2.04
Saccharomyces cerevisiae K1	8.26 ± 0.75	0.10 ± 0.06	1.21
Saccharomyces cerevisiae 22	7.66 ± 0.98	0.06 ± 0.01	0.78
Saccharomyces cerevisiae 26	7.29 ± 0.41	0.43 ± 0.18	5.90
Saccharomyces cerevisiae 5	4.64 ± 0.38	0.15 ± 0.84	3.23
Saccharomyces cerevisiae DHW 12	5.82 ± 0.94	0.25 ± 0.05	4.30
Saccharomyces cerevisiae 6815	11.86 ± 1.96	0.03 ± 0.00	0.25
Saccharomyces cerevisiae 32	4.72 ± 1.53	0.06 ± 0.13	1.27
Saccharomyces cerevisiae 34	5.49 ± 0.13	0.11 ± 0.01	2.00
Saccharomyces cerevisiae 43	8.29 ± 0.48	0.16 ± 0.09	1.93
Saccharomyces bayanus 1A18	5.35 ± 1.32	0.06 ± 0.02	1.12
Saccharomyces pasterianus 4	6.05 ± 0.03	0.03 ± 0.00	0.50
Saccharomyces diastaticus 27	1.47 ± 0.33	0.29 ± 0.18	19.73
Saccharomyces bailii var. *bailii* 46 (24 G)	7.46 ± 0.44	0.08 ± 0.00	1.07
Schizosaccharomyces pombe 57 (24 A)	3.04 ± 0.65	0.13 ± 0.04	4.28
Kloeckera apiculata 58	10.15 ± 0.38	0.03 ± 0.00	0.30
Rhodotorula glutinis var. *glutinis* 60	0.23 ± 0.02	0.05 ± 0.02	21.74
Candida membranaefaciens (No.1) 61	3.21 ± 0.17	0.13 ± 0.04	4.05
Candida tropicalis Cappo (571) 64	6.04 ± 0.03	0.05 ± 0.01	0.83
Candida lipolytica 66	13.11 ± 1.57	0.05 ± 0.03	0.38
Hansenula anamola var. *anamola* IB (T) 69	8.96 ± 1.01	0.11 ± 0.05	1.23
Kluyveromyces dobshankii 2A73	10.57 ± 0.60	0.13 ± 0.08	1.23

Beyatlı et al. (1997) reported that the percentage of PHB was found 2.7%, 5.0% and 5.6% in *Candida tropicalis* CT, *Saccharomyces cerevisiae* SC and *Kluyveromyces lactis* KL, respectively (20). The yield of PHB of these strain was observed to be low according to our strains. In addition, the percentage of PHB in our isolates was found to be lower than the percentage of PHB in the yeast strains obtained from A.U.Z.F. Among isolates, the maximal PHB was determined in *Kloeckere apiculata* Z3. The PHB content in cell of this strain reached 16.67% of dry cell weight. The percentage PHB in cells is normally low, between 1 and 30%, but under controlled fermentation conditions for carbon excess and nitrogen limitation, over production of polymer can be encouraged and yields increase to about 70% of dry cell weight (21). According to Huang et al. (1999), *Zoogloea* sp. (Z5-611) was produced 61.86% in cell (22). On the basis of data obtained in the present work, the content of PHB of yeast strains was found to be much lower than *A. eutrophus* that is a industrial producer of PHB synthesis (23) and other bacteria.
In most microorganisms PHB is synthesized and intracellularly accumulated under unfavourable growth conditions such as limitation of nitrogen, phosphorus, magnesium, or oxygen in the presence of excess carbon source. Selection of a microorganism for the industrial production of PHB should be based on several factors including the cell's ability to utilize an inexpensive carbon source, growth rate, polymer synthesis rate, and the maximum extent of polymer accumulation. In other words, it is necessary to determine most suitable of carbon and nitrogen sources to microorganism to decrease the production cost at minimum level and increase the production. In our study, the capacity for PHB synthesis was tested in strains of *Rhodotorula glutinis* var. *glutinis* 60 and *Saccharomyces diastaticus* 27 which produced the maximum PHB percentage during growth on media with different carbon and nitrogen sources. The PHB content in *Rhodotorula glutinis* var. *glutinis* 60 reached 2.41% of dry cell weight in YEPD medium supplemented with arabinose, 10.45% with sucrose and 21.95% during growth on a medium with mannitol. Among all carbon sources, the maximum PHB yield was found in cell grown with mannitol (21.95%), but increasing of PHB in strain is not important. As can be seen from Table 4, in *Rhodotorula glutinis* var. *glutinis* 60, the maximum PHB content was found in cell grown with $(NH_4)_2SO_4$ as the nitrogen source and the strain 60 reached 19.44% of dry cell weight. The PHB content was much lower when other nitrogen sources were used. The production of PHB in this strain was found to be 21.74% in control medium. Generally, for *Rhodotorula glutinis* var. *glutinis* 60 the effect of carbon and nitrogen sources was not observed on PHB synthesis.

Table 4. The production of PHB of the *Rhodotorula glutinis* var. *glutinis* 60 strain on media with different carbon and nitrogen sources.

Carbon and Nitrogen Sources	Dry Cell Weight **(g/l)**	[a]**PHB (g/l)**	[b]**Yield of PHB (%)**
Mannitol	0.41 ± 0.02	0.09 ± 0.04	21.95
Sucrose	0.67 ± 0.12	0.07 ± 0.01	10.45
Arabinose	2.49 ± 0.41	0.06 ± 0.01	2.41
L-Cystein	4.59 ± 0.90	0.07 ± 0.01	1.53
$(NH_4)_2SO_4$	0.36 ± 0.01	0.07 ± 0.02	19.44
Glycine	0.35 ± 0.02	0.06 ± 0.02	17.14
Protease Peptone	0.48 ± 0.12	0.08 ± 0.03	16.67
DL-Tryptophan	0.39 ± 0.01	0.07 ± 0.01	17.95
Control	0.23 ± 0.02	0.05 ± 0.02	21.74

Table 5. The production of PHB of the *Saccharomyces diastaticus* 27 strain on media with different carbon and nitrogen sources.

Carbon and Nitrogen Sources	Dry Cell Weight **(g/l)**	[a]**PHB (g/l)**	[b]**Yield of PHB (%)**
Mannitol	0.31 ± 0.10	0.04 ± 0.00	12.90
Sucrose	0.80 ± 0.01	0.08 ± 0.01	10.00
Arabinose	0.21 ± 0.04	0.04 ± 0.00	19.05
L-Cystein	4.43 ± 1.46	0.08 ± 0.01	1.81
$(NH_4)_2SO_4$	1.19 ± 0.22	0.07 ± 0.00	5.88
Glycine	2.50 ± 0.11	0.05 ± 0.00	2.00
Protease Peptone	2.11 ± 0.02	0.06 ± 0.01	2.84
DL-Tryptophan	0.24 ± 0.05	0.06 ± 0.02	25.00

Control	1.47 ± 0.33	0.29 ± 0.12	19.73

In different of carbon sources, the percentage of PHB in *Saccharomyces diastaticus* 27 was found to be 19.05% with arabinose, 10.0% with sucrose and 12.90% of dry cell weight during growth on a medium with mannitol. Among all carbon sources, the maximum PHB yield was found in cell grown with arabinose (19.05%). In *Saccharomyces diastaticus* 27, the maximal PHB content was found in cells grown with DL-tryptophan as the nitrogen source and the strain capable of PHB accumulation up to 25.0% of dry cell weight. The PHB content wasn't high in this strain grown on L-cystein, $(NH_4)_2SO_4$, glycine and protease peptone. The production of PHB in this strain was found to be 19.73% in control medium (Table 5).

According to the results of our work, the production of PHB in yeasts was observed to be much lower than in the prokaryotic microorganisms. It has been shown that the maximum PHB content depend upon the type of culture and species. At the end of this study it was observed that isolates such as *Saccharomyces diastaticus* 27 (19.73%), *Rhodotorula glutinis* var. *glutinis* 60 (21.74%) and *Kloeckera apiculata* Z3 (16.67%) with the highest PHB yield had similarity with the PHB yield of procaryotes. Although it was thought that it was possible to increase the PHB yield with different carbon and nitrogen sources, it was observed that the tested carbon and nitrogen sources did not cause any increase. However, *Saccharomyces diastaticus* 27 in DL-tryptophan medium showed the best result in PHB yield in comparison to the other isolates (25%). In the same medium *Rhodotorula glutinis* var. *glutinis* 60 strain didn't report any increase in the PHB yield. As a result it is believed that the different carbon and nitrogen sources produced different results in different species.

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