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MOLECULAR IDENTIFICATION OF YEAST ISOLATED FROM DIFFERENT KOMBUCHA SOURCES

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The Kombucha microbial consortium consists mainly of yeast and lactic bacteria which ferment the carbohydrates and release ethanol, respectively lactic acid, as well as of acetic bacteria which lead to the production of different organic acids. The symbiotic consortium of bacteria and yeast (SCOBY) from Kombucha have been used in the past years in the production of functional food, including tri-biotic products made of fermented pollen. The goal of the research it was to characterize, by PCR-ITS RFLP technique, the yeast biodiversity of SCOBY/Kombucha used in the preparation of the new tri-biotic functional product. Nine yeast isolates from Kombucha fermented product made of green tea have been used. Conserved region of yeast have been amplified with universal primers (ITS 1 and ITS4). Different restriction profiles have been obtained after enzymatic digestion with *Hinf*I, *Hae*III, *Hha*I. PCR amplified fragments were of different sizes (450bp, 600bp, 680bp and 800bp). After the use of restriction enzymes four different ITS-RFLP patterns have been obtained and compared to reported results. The high level identity (99%) confirm that the isolates type I belong to *Dekkera bruxellensis* species. The future investigations will be to assess the potential probiotic properties of yeast isolates.

Keywords: Kombucha, yeast, PCR-ITS RFLP, functional food

Introduction

Kombucha is a traditional non-alcoholic beverage originating in the Orient, prepared basically from green or black tea fermented by a microbial consortia known as SCOBY (*Symbiotic Consortia of Bacteria and Yeast*). These consortia have a high ecological and technological plasticity, being resistant to other microbial contamination (Jayabalan et al., 2014). In the past years Kombucha SCOBY has been used in the preparation of functional food staring from other substrates than the infused tea, like coffee (Watawana et al., 2015) or milk (Malbasa et al., 2009). Recently, the bee pollen has been fermented with Kombucha consortia to increase the biodisponibility of the active pollen's

compounds and to add to the final product specific active compounds from Kombucha like organic acids, vitamins, peptides, polyphenols (Marsh et al., 2014).

The Kombucha SCOBY type consist mainly of yeast and lactic bacteria which ferment the carbohydrates and release ethanol, respectively lactic acid, as well as of acetic bacteria which lead to the production of different organic acids, especially acetic acid, but also gluconic or galacturonic acid, propionic acid, etc (Marsh et al., 2014).

According to Marsh et al. (2014) the microbial biodiversity in Kombucha consortium depends on the fermentation length, the geographic origin of the SCOBY, but also isolation layer (Kombucha pellicle/membrane or "soup").

Related to the yeast biodiversity in Kombucha, it has been assumed that is higher than the bacterial one. The identified yeast belongs to very diverse gender like *Zygosaccharomyces* (Marsh et al., 2014; Teoh et al., 2004; Jayabalan et al., 2008), *Candida* (Mayser et al., 1995; Chakravorty et al., 2016), *Kloeckera/Hanseniaspora* (Marsh et al., 2014; Chakravorty et al., 2016), *Torulaspora* (Teoh et al., 2004), *Pichia* (Velićanski et al., 2014; Chakravorty et al., 2016), *Brettanomyces/Dekkera*, *Saccharomyces* and *Saccharomycoides* (Markov et al., 2001; Marsh et al., 2014).

Microbial counts have been reported to be greater in the tea broth than the cellulosic pellicle (Goh et al., 2012). Generally, the yeast population in Kombucha rise to 10^4-10^6 CFU/ml after approximately 10 days of fermentation; higher levels have been counted in the liquid phase ("the soup"). Meanwhile, while the acetic bacteria lead to the decrease of the pH in the medium by the formation of acetic acid, the yeast levels decrease progressively (Marsh et al., 2014).

The goal of the research it was to characterize, by molecular tools, some yeast isolates from SCOBY/Kombucha used in the preparation of a new tri-biotic functional product made of fermented pollen.

Materials and methods

Biologic materials

The isolation source for yeast was pellicle/membrane (coded as KGM) and "soup" (coded as KGS) from Kombucha obtained on green tea infusion (10% sugar) fermented with SCOBY provided by Laboratoarele Medica, Otopeni, Romania. The microbial diversity of the employed SCOBY has never been characterized; its industrial use has been based on literature reports on different Kombucha SCOBY sources (Dufrense and Farnworth, 2000; Chen and Liu, 2000; Marsh *et al*., 2014). This SCOBY, cultivated under constant fermentative conditions and substrate, is used on industrial scale for the production of a new tri-biotic functional product based on fermented pollen; the product has been notified and certified for commercialisation

Yeast isolation and quantification

The yeast have been isolated on yeast extract peptone dextrose agar (YEPD) medium supplemented with chloramphenicol (10 ml/L) as described by Matei F. et al. (2011). Before the inoculation, for the "soup" has been applied decimal dilutions

according to the haematological counting method; in the case of the pellicle, the "mother solution" has been obtained by washing with sterile distilled water. After inoculation, the yeast has been cultivated at 27ºC during 48-72 hours. To quantify the viable yeast, the developed colonies have been counted and used to calculate the CFU/ml.

Yeast identification

In a preliminary step, the yeast isolates have been described for their cultural characteristics on solid media and microscopically for their cell shape or budding. Further, all the isolates have been subject to DNA extraction and molecular identification by PCR-ITS-RFLP technique. Yeast biomass obtained after cultivation at 27ºC during 24-48 hours has been subject to DNA extraction by the use of a kit (ZR Fungal DNA MiniPrep, Zymo Reserch). The DNA purity has been determined with a SpectraMax® QuickDrop™ (Molecular Devices) Further, conserved region of yeast have been amplified with universal primers (ITS 1: 5´-TCCGTAGGTGAACCTGCGG-3´ and ITS4: 5´-TCCTCCGCTTATTGATATGC-3´) by the use of a MultiGene termocycler. The amplification parameters were: denaturation at 94°C/1.5 min; hybridization at 55.5ºC/1.5 min; elongation at 72ºC/2 min with a final elongation of 10 min. Starting from the Esteve-Zarzoso *et al.* (1999) results and recommendation, the amplified products have been digested with different restriction enzymes (*Hinf*I, *Hae*III, *Hha*I, Thermo Fisher Scientific) during 1 hour at 37ºC, according to manufacturer's protocol. The amplification and digestion results have been visualized under UV (254 nm) after electrophoresis in 2% agarose gel (90V/ 60 minutes. All fragments size has been approximated according to the gel migration and by comparison against *the* known size DNA marker (GeneRuler 100bp DNA Ladder, Thermo Fisher Scientific); no theoretical program has been employed. Meanwhile, the PCR products of different sizes have been sequenced (Microsynth, Austria) and the obtained sequences have been analyzed by the use of EMBL database (www.srs.ebi.ac.uk). Also, theoretic digestion has been applied by the use of the Harry Mangalam's tacg 4.3 program (http://biotools.umassmed.edu/tacg4).

Results and discussion

The main purpose of the work it was to isolate and identify yeast associated to an industrial source of Kombucha in the membrane (pellicle) and in the "soup" (suspension). During the experiments, as secondary goal, it was to quantify the number of yeast in both media. By the use of classical techniques of quantification (on-plate cultivation) it has been shown that in the first 6 fermentation days the yeast level varies (on average) between $1.7x10^2$ CFU/ml in the membrane and $2.1x10^3$ CFU/ml in the suspension. From the 10^{th} day of the fermentation by the end of the process (18^{th} day) in both media, the yeast concentration remains practically constant at 10^5 -10^6 CFU/ml (table 1 and figure 1), which is in accordance with the results obtained by Marsh et al. (2014).

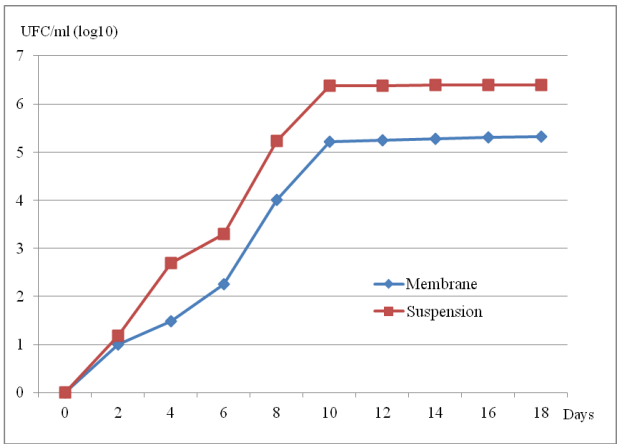

Figure 1. Yeast population evolution in the membrane and in the suspension during Kombucha fermentation

Table 1. Yeast viable cells evolution during green tea Kombucha fermentation

Kombucha layer	Day 6	Day 10	Day 18
Membrane	$1.77 \times 10^2 \pm 0.05$	$1.66 \times 10^5 \pm 0.03$	$2.11 \times 10^5 \pm 0.01$
Suspension	$2.01 \times 10^3 \pm 0.61$	$2.41 \times 10^6 \pm 0.67$	$2.5 \times 10^6 \pm 0.62$

Note: data represents the means of viable cells quantified in three different Petri dishes

Taking into account the macroscopic observations of the yeast colonies in the plates, the isolation step has been finalized with a total of 9 isolates, respectively 6 from the suspension (codified as KGS1 to KGS6) and 3 from the membrane (codified as KGM1 to KGM3). Under the optical microscope, the discriminate characters were the cell shape and size and budding; also, macroscopic characters of the colonies on YPG have been taken into account; finally, the nine yeast isolates have been grouped in 4 different typologies (table 2).

Table 2. Different typology of yeast isolated from Kombucha SCOBY

Yeast typology	Colonies characterisation on YPG medium	Cells characterization under optical microscopy
I	small, even, white-cream color	oval/elongated shape; polar budding
II	small, umbonate, white-cream color	ovoidal, long-ovoidal; polar budding
III	medium sized, circular shape, even, yellow-cream color	oval shape; no budding
IV	medium sized, circular shape, even, yellow-cream color	round shape; no budding

All nine isolates have been subject to molecular identification by PCR-ITS-RFLP coupled with sequencing. The DNA extraction was successful from the very beginning and it has been obtained pure DNA with concentration varying from 220 to 510 $ng.\mu L^{-1}$. The total DNA has been amplified with universal primers ITS1 and

ITS 4. As seen in the figure 2 there were obtained amplicons with four different sizes, respectively 450 bp, 600 bp, 680 bp and 800 bp. These results may be correlated to the macroscopic and microscopic data which led to four different typologies among the yeast isolates.

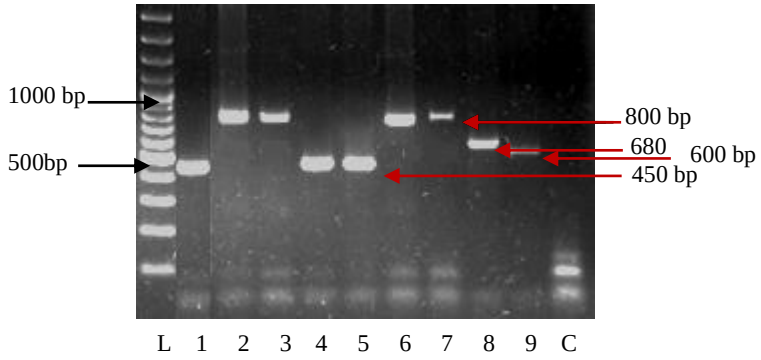

Figure 2. Amplified fragments with universal primers ITS 1 and ITS 4 of total yeast DNA isolated from Kombucha (L- GeneRuler 100bp DNA Ladder; 1-KGM1; 2- KGS1; 3-KGS2; 4-KGM2; 5-KGM3; 6-KGS3; 7-KGS4; 8-KGS5; 9-KGS6; C- control with primers mix)

According to Esteve-Zarzoso et al. (1999) results and recommendation, the amplified products have been digested with different restriction enzymes (*Hinf*I, *Hae*III, *Hha*I).

By enzymatic digestion with ***Hinf* I** (figure 3; table 3) have been obtained four different restriction profiles (coded A, B, C, D following the procedure of Martinez-Culebras and Ramon, 2007), respectively: A (2 different fragments) for KGM1, KGM2 and KGM3; B (3 different fragments) for other four strains (KGS1, KGS2, KGS3 and KGS4); C (2 different fragments) for KGS5; D (2 different fragments) for KGS6.

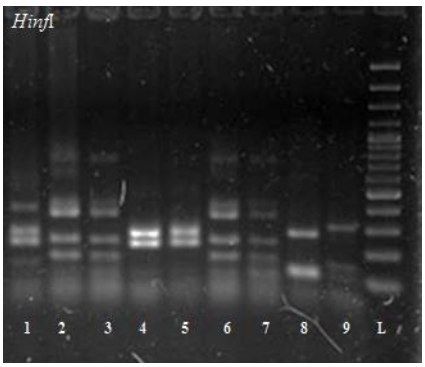

Figure 3. Enzymatic digestion with ***Hinf* I** of amplified total DNA of Kombucha yeast isolates (1-KGM1; 2- KGS1; 3-KGS2; 4-KGM2; 5-KGM3; 6-KGS3; 7-KGS4; 8-KGS5; 9-KGS6; L- GeneRuler 100bp DNA Ladder)

In the case of the enzymatic digestion with ***Hha*I** (figure 4; table 3) have been obtained the following four different restriction profiles (coded A, B, C, D): A (2 different fragments) for KGM1, KGM2 and KGM3; B (3 different fragments) in the case of KGS1, KGS2, KGS3 and KGS4; C (3 different fragments) for KGS5 and D (one fragment considered as doubled profiled) while for KGS6.

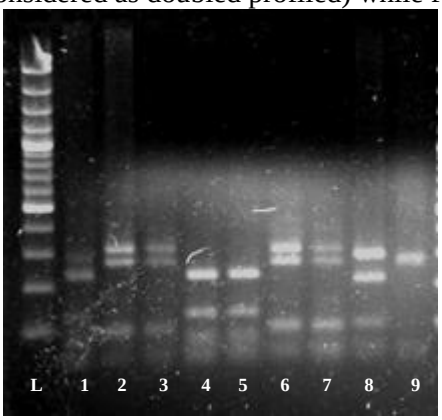

Figure 4. Enzymatic digestion with ***Hha*I** of amplified total DNA of Kombucha yeast isolates (L- GeneRuler 100bp DNA Ladder; 1-KGM1; 2- KGS1; 3-KGS2; 4-KGM2; 5-KGM3; 6-KGS3; 7-KGS4; 8-KGS5; 9-KGS6)

The last performed digestion it was the one with ***Hae*III** (figure 5; table 3). Only for three strains (KGM1, KGM2 and KGM3) have been obtain a clear restriction profile, code as A, having 2 fragments. For the other strains have been obtained a profile similar with the amplified amplicon (coded B, C, D in table 3).

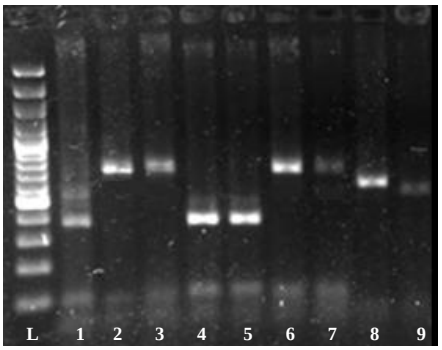

Figure 5. Enzymatic digestion with ***Hae*III** of amplified total DNA of Kombucha yeast isolates (L- GeneRuler 100bp DNA Ladder; 1-KGM1; 2- KGS1; 3-KGS2; 4-KGM2; 5-KGM3; 6-KGS3; 7-KGS4; 8-KGS5; 9-KGS6)

The ITS-RFLP patterns of the yeast isolates after enzymatic digestion with all the three restriction enzymes are presented in Table 2. It can be noticed the presence four patterns corresponding to previous data obtained only by ITS amplification.

Table 3. ITS-RFLP pattern of the amplicons digested with three different restriction enzymes

Kombucha yeast isolates	PCR products (pb)	Restriction profiles (pb)			ITS-RFLP pattern type
		*Hae*III	*Hinf*I	*Hha*I	
KGM_1	450	A	A	A	I (AAA)
KGS_1	800	B	B	B	II (BBB)
KGS_2	800	B	B	B	II (BBB)
KGM_2	450	A	A	A	I (AAA)
KGM_3	450	A	A	A	I (AAA)
KGS_3	800	B	B	B	II (BBB)
KGS_4	800	B	B	B	II (BBB)
KGS_5	680	C	C	C	III (CCC)
KGS_6	600	D	D	D	IV (DDD)

For each type of the ITS-RFLP pattern, the ITS1/ITS4 amplified fragments have been sent for sequencing by Microsynth (Austria). The amplified fragment ITS1-5.8S-ITS4 has been sequenced in both directions direction. By the use of Multiple Sequence Alignment, Clustal Omega tool, (http://www.ebi.ac.uk/Tools/msa/clustalo/) the complete fragment ITS1-5.8S-ITS4 has been identified (100%) for the strain KGM1, a type I yeast profile (figure 5).

TCCGTAGGTGAACCTGCGGAAGGATCATTACAGGATGCTGGGCGCAAGCCCGTGCAGACACGTGGAT
AAGTAAGGATAAAAATACATTAAATTTATTTAGTTTTAGTCAAGAAAGAATTTTAAAACTTTCAACA
ATGGATCTCTTGGTTCTCGCGTCGATGAAGAGCGCAGCGAATTGCGATACTTAATGTGAATTGCAGA
TTTTCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTT
TGAGCGTCATTTCCTTCTCACTATTTAGTGGTTATGAGATTACACGAGGGTGTTTTCTTCAAAGGAA
AGAGGGGAGAGTGAGGGGATAATGATTTAAGGTTTCGGCCGTTCATTATTTTTTCTTCTCCCCCAGT
TATCAAGTTTGACCTCAAATCAGGTAGGAGGACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA

Figure 5. Complete sequence ITS1-5.8S-ITS4 of KGM1 yeast isolate from Kombucha ITS1 primer (red) and ITS4 primer (blue)

The sequence analysis has been performed by comparison with sequence present in international database, by the use of Basic Local Alignment Search Tool https://blast.ncbi.nlm.nih.gov/Blast.cgi. In the case of the type I yeast profile (represented by the strain KGM1) it has been obtained a 99%-100% identity with the specie *Dekkera/Brettanomyces bruxellensis*. The isolates type I (KGM1, KGM2 and KGM3) have been identified as *Dekkera/Brettanomyces bruxellensis.*

Lower identity levels (70-80%) have been obtained in the case of the other three yeast profiles types (II-IV) with species which have not been yet reported in Kombucha SCOBY (*Bullera sp., Sporidiobolus sp.* and its telomorph *Sporobolomyces sp.*). Further investigation should be performed to clarify their identity. *Dekkera bruxellensis* JQ327829.1.

Conclusions

Kombucha beverages, originating in ancient times in far Orient, have been paid a special attention for the production of functional food in the last decade. In this respect, the characterisation of its microbial diversity is a must. In our effort to search for the presence of probiotic yeast in Kombucha tri-biotic product (pollen fermented with Kombucha) we have isolated nine yeast strains having four different ITS-RFLP patterns after the digestion with *Hinf*I, *Hae*III and *Hha*I. The high level identity (99%) confirm that the isolates type I belong to *Dekkera bruxellensis* specie. However, to validate the results with lower identity levels is required to repeat the sequencing. Despite the expectations and other reported results (Chakravorty et al., 2016; Jayabalan et al., 2014; Marsh et al., 2014), among our isolates no *Saccharomyces* sp. has been isolated and identified. In this respect, most probably a Kombucha suspension and membrane total DNA approach should be taken into account and multiplex-PCR may be applied with specific primers. A special attention will be given in the future to the dynamic of the yeast during the fermentation by a Q-PCR approach.

Acknowledgments

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