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High Throughput Sequencing as a novel quality control method for industrial yeast starter cultures

The use of pure starter cultures is largely responsible for the great success of today's fermentation products market. With their implementation, fermentations (e.g., beer fermentation) have become predictable, efficient, controllable and reproducible. The pureness of the applied starter culture is therefore of great interest for obtaining these advantages. Many protocols have been applied for quality control of the pureness of these cultures, and they have improved over the last century. The current state of the art of detecting interfering microorganisms consists of the use of selective media or targeted approaches via Real-Time PCR. These methods are time consuming and require suspicion of the identity of potential interfering microbe(s). The use of High Throughput Sequencing, however, offers the ability to apply a non-targeted approach for the detection of interfering spoilers in the applied case of spoilage yeasts. Here we used the 26S rDNA D1/D2 region of chromosome XII to verify the pureness of yeast cultures applied in brewing, wine and special beer fermentations. The results show that it is possible to detect differing species in supposedly pure yeast cultures by application of the new method. Some strains showed potential traits of intraspecific hybridization, horizontal gene transfer or syntrophic cultures, which interfered with the results. The 26S rDNA D1/D2 region showed to be discriminative for only some species, indicating the need to additionally apply more discriminative regions like ITS1. Furthermore, we propose a more comprehensive and powerful database, consisting of highly validated and identified cultures, that has to be built up to improve sequencing results.

Descriptors: HTS, quality control, yeast, fermentation, pure cultures

1 Introduction

Pure and defined microbiological starter cultures, also called defined strain starters (DSS), are a valuable tool for predictive and controllable industrial fermentations [1]. DSS are defined as consisting of one or more strains of one or more species [1; 2]. Since the implementation of pure brewing yeast cultures by Emil Christian Hansen in 1883 [3], one of the first pure culture fermentation approaches, the global amount of fermentation volume over all industrial applications has increased rapidly. The total value of the global fermentation products market reached $ 149,469 million in 2016 and is predicted to grow further to $ 205,465 million by the year 2023 [4].

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Pitching a pure culture of starter microorganism into a defined media results in a predictive process with a defined product [1]. Interference by other microorganisms, whether other species, or even just other strains from the same species will change the result of the fermentation. This will result in loss of product, inefficiency and potentially complete spoilage of batches, which in the worst case must be discarded [5]. Yeast strains as valuable fermenting microorganisms are used for many industrial fermentations. Purity of the applied yeast strains is important for processes such as wine, beer, and ethanol production, as well as to produce proteins in the biopharma industry [4; 6; 7]. For as long as DSS have been implemented, the ability to control the purity of yeast strains has been indispensable. In the last centuries and decades, the level of quality control of fermentations started to improve with the refinement of the microscope, became more sufficient with various selective enrichment cultivation media and physiological tests, and reached a high point with the implementation of Real-Time-PCR systems [8]. All these methods were developed to identify potentially differing microbes, which interfere with the purity of a culture or a culture fermentation. Every novel method has lowered the level of detection and increased the purity of fermentations.

As an example for potential detection of interfering spoilage yeasts, one may look at pure yeast cultures for beer fermentations. Analysis by microscope is restricted to a small amount of sample at a time, and high-level experience and knowledge is needed to identify

potentially different cell morphologies. Moreover, low levels of contamination by spoilage yeast in a crowded yeast sample can be detected only with great difficulty [9]. When using selective media to identify potential interfering yeasts in the fermentation process, the main culture is suppress by defined additives (e.g. antimycotics). If this additive also suppresses the interfering microorganism, or if the desired microorganism is resistant against the additive, a false negative or false positive result may be the outcome. Real-Time-PCR is tied to primers and probes, which are used to detect spoilers by targeted approaches. If a target is unknown, spoilage yeasts can potentially be detected by applying certain numbers of targeted approaches simultaneously in a process called multiplex systems. However, if the interfering spoilage yeast is not on the target list, high costs and a failed detection is the result [10].

In contrast, High Throughput Sequencing (HTS) can be applied as an untargeted approach of identifying potential spoilage yeasts in pure yeast starter cultures without prior knowledge of the identity of the potentially contaminating organism. For our application, the large subunit 26S rDNA D1/D2 region was chosen for the first approach as it contains variable species-specific sequences [11]. Prior to HTS, Sanger sequencing was the state of the art sequencing technique for many years. Now, HTS offers the ability to sequence multiple amplicons of one gene fragment, in contrast to classical Sanger sequencing. The latter sequenced the most frequently occurring fragment, which could then be linked to one species at a time. Less frequently occurring sequences were mostly not visible and could therefore go undetected [12]. HTS enables sequencing of differing amplicons, providing the opportunity to detect multiple species in the same sample at the same time [13; 14]. Recent results from medical research also show that it might be possible in the future to differentiate between strains of a single species as well [11; 15].

Table 1 Applied yeast strains of this study, strain abbreviation, species and applied fermentations purpose

Strain abbreviation	Species	Fermentation purpose
TUM 193	*Saccharomyces pastorianus*	Lager yeast
TUM 68	*Saccharomyces cerevisiae*	Wheat beer yeast
TUM 127	*Saccharomyces cerevisiae*	Wheat beer yeast
TUM 177	*Saccharomyces cerevisiae*	Koelsch, Ale yeast
TUM 184	*Saccharomyces cerevisiae*	Alt, Ale yeast
TUM 506	*Saccharomyces cerevisiae*	Ale yeast
TUM 211	*Saccharomyces cerevisiae*	Ale yeast
TUM 511	*Saccharomyces cerevisiae*	Ale yeast
TUM 381	*Saccharomyces cerevisiae*	Trappist, Ale yeast
TUM T 90	*Torulaspora delbrueckii*	Special beers
TUM 523	*Hanseniaspora uvarum*	Banana wine
TUM 536	*Schizosaccharomyces pombe*	Special beers
TUM Bretta 1	*Brettanomyces bruxellensis*	Lambic yeast
TUM SL 17	*Saccharomycodes ludwigii*	Low alcohol wheat beer yeast
TUM V 1	*Saccharomyces cerevisiae*	Wine fermentation
TUM V 12	*Saccharomyces cerevisiae*	Wine fermentation
TUM V 2	*Saccharomyces cerevisiae*	Wine fermentation
TUM 5-2-1	*Kazachstania unispora*	Spoilage yeast

The created fragment sequences are further processed to OTUs (Operational Taxonomic Units) at a length of about 150–250 bases and compared to publicly available databases (such as NCBI; see section Bioinformatics). As the large subunit 26S D1/D2 region is of interest due to its relatively high species/strain specificity, many reference sequences are already available. To generate a proof of this concept, HTS was applied as a new untargeted quality control tool for purity of yeast DSS. This HTS approach was applied to a total of 20 pure yeast samples and one pooled sample (nine commercially available pure *Saccharomyces* brewing yeast cultures, five pure non-*Saccharomyces* yeast cultures applied for special beers, three *S. cerevisiae* wine cultures and one isolated environmental spoilage yeast culture). The pooled sample was created to test the recovery of three species out of a pool of varying unknown species. The large subunit 26S rDNA D1/D2 region was used in this proof of principle test, but any other species-specific region such as ITS1 could also be applied [11].

2 Materials & Methods

2.1 Applied yeast strains

Table 1 lists the yeast strains that were used in this study. Pure strains were cultivated on wort agar slopes for 72 hours at 28 °C and stored in a sterile environment at 2–4 °C. The strains were subcultured at intervals of one month.

For the pooled sample, a mixture of TUM 211, TUM 523, TUM 5-2-1 and spontaneous growing yeast species was set up. For this purpose, samples of the pure cultures were added to a sample of 50 ml of spontaneously fermented wort at 12 °P (cool wort was left open prior to inoculation at an open window for one day). Fermentation was performed for 2 days at 28 °C. 1 ml of the sample was taken sterile, and DNA was extracted as described in paragraph 2.2..

2.2 DNA extraction & High-Throughput Sequencing

Samples were taken from wort agar slopes with sterile inoculation loops and transferred into 1.5 mL Eppendorf tubes. 1 ml of the pooled wort sample was taken sterile and transferred into a 1.5 mL Eppendorf tube. DNA extraction was performed using the Qiagen DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Cleaned and extracted DNA was then used as a template to amplify the D1/D2 domain of the 26S rRNA gene using NL1 (5`-GCATATCAATAAGCGGAGGAAAAG-3`) and NL4 (5`-GGTCCGTGTTTCAAGACGG-3`) (95 °C/5 min; 35 cycles of 95 °C/30 s, 52 °C/60 s; 72 °C/60 s; 72 °C/10 min), primers were equipped with a binding region for Illumina index sequences [16]. In a second PCR reaction, successfully amplified samples were equipped with a unique combination of Illumina index sequences. For the construction of the Illumina libraries, we used the cleaned amplicons, which were pooled to equal molarity (100 ng). A preparative gel electrophoresis was used for size-selection of the Illumina libraries. High-Throughput Sequencing (HTS) was performed on

an Illumina MiSeq using v2 (2*250 bp, 500 cycles, maximum of 20 mio reads) chemistry.

2.3 Bioinformatics

Processing of sequences was performed with the VSEARCH v2.4.3 suite [17] and cutadapt v1.14 [18]. Only forward reads (approximately 234-bp long) were used for the analysis, due to low quality of the reverse reads preventing paired-end merging. Forward primers were removed with cutadapt, using the "discard_untrimmed" option to discard sequences for which the primer sequence was not reliably detected at >= 90% identity. Quality filtering was performed with the "fastq_filter" in VSEARCH, keeping sequences with zero expected errors ("fastq_maxee" 1). Sequences were dereplicated with "derep_fulllength", first at the sample level, and then as one entire fasta file. Chimeric sequences were filtered out using "uchime_denovo". "cluster_size" was used to cluster the remaining sequences into OTUs at 97% identity and create a contingency table of counts of reads per OTU per sample. To reduce noise, read counts were eliminated from the OTU table which were less than 0.01% of the total numbers of reads for their corresponding samples (see JAMP v3, https://github.com/VascoElbrecht/JAMP/). OTUs were BLASTed against the GenBank nucleotide database (nt) in Geneious (v9.1.7 – Biomatters, Auckland – New Zealand) program Megablast with default parameters. The resulting csv file, which included hit descriptions, taxonomy, Hit-%-ID value, and bit score, was exported from Geneious and combined with the OTU table. Graphs were created with OriginPro 2018b (OriginLab Corporation).

3 Results and Discussion

The following shows the results of the HTS sequencing of the 26S rDNA D1/D2 region of 20 pure yeast cultures and one pooled sample. The varying numbers of reads reflect varying levels of amplification success of the PCR reactions performed prior to sequencing. All percentage shares are calculated according to the number of reads for the corresponding sample. The results do not represent quantitative detection of species in the sample, they represent the amount of amplification and can therefore not give a defined rate of contamination. All applied OTU's were BLASTed against the NCBI GenBank nucleotide database (nt). Resulting species/strains can be found with respective accession number and percentage of pairwise identity in table 2. Accession number can be used to identify according sequences, projects and species names (https://blast.ncbi.nlm.nih.gov/Blast.cgi). As shown in table 2, some sequences of the database were labelled unspecific as uncultured fungus and uncultured *Saccharomycetaceae*, which indicates that an actual database for the here applied experiments needs to be augmented in order to produce reliable results. However, the database was sufficient for this first proof of principle.

Table 2 OTU sequence results for according species and accession numbers

Pairwise identity [%]	Accession number	Species	OTU ID in this project
100	CP033481	*S. cerevisiae*	1
100	MH443765	*H. uvarum*	4
100	KY296084	*Schizosaccharomyces pombe*	7
100	MK034127	*T. delbrueckii*	3
98.2	JX409606	*Uncultured Saccharomycetaceae*	18
100	KY109478	*Saccharomycodes ludwigii*	6
100	KF908878	*Dekkera anomala*	11
97.9	KY107593	*Brettanomyces anomalus*	99
98.3	KF810069	Uncultured yeast isolate ZB01142638	26
97.4	KF810090	Uncultured yeast isolate ZB05224068	28
99.1	MG525064	*Kazachstania unispora*	15
100	MG927742	Uncultured fungus	10
100	MG773367	*Wickerhamomyces pijperi*	9
100	KY558364	*Kluyveromyces dobzhanskii*	12

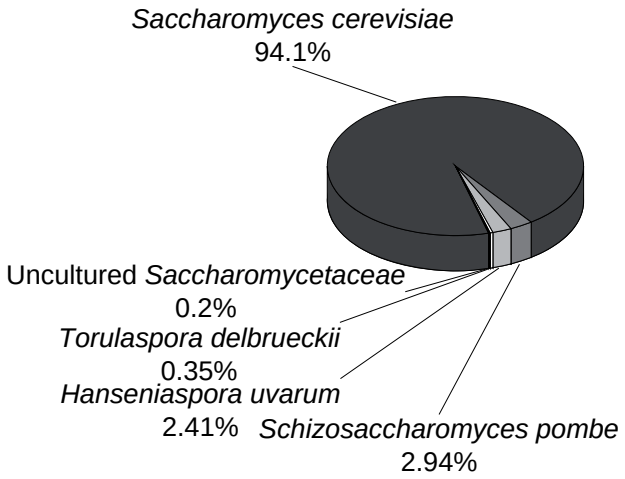

Fig. 1 Composition of OTU's displayed in percent, detected for strain TUM 184 *Saccharomyces cerevisiae* culture (percentages result from total of 8946 reads)

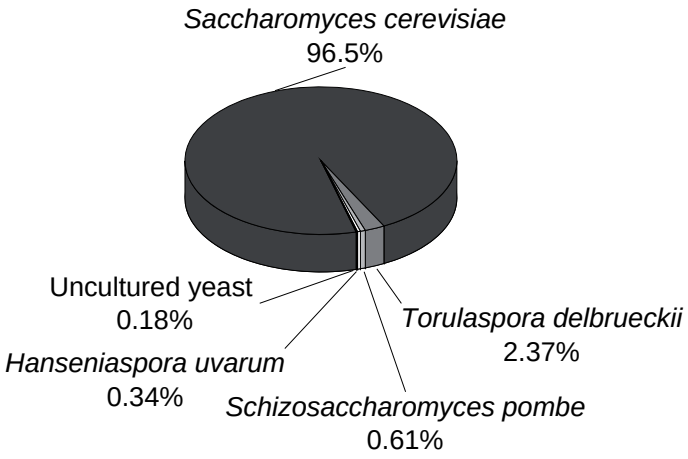

Fig. 2 Composition of OTU's displayed in percent, detected for strain TUM 68 *Saccharomyces cerevisiae* culture (percentages result from total of 7251 reads)

Completely pure cultures of single species were expected to show 100 % of one species. Sample TUM 211, TUM 381, TUM 177, TUM 506, TUM 511, TUM V12 and TUM V2 showed these expected results. A total of 100 % of OTU 1 (Table 2), *Saccharomyces cerevisiae* sequence were detected. TUM 211, TUM 381, TUM 177, TUM 506, TUM 511 are commercially available strains which are used to produce top fermented beers [19]. TUM V12 and TUM V2 are commercially available wine strains. The results indicate that the cultures were of pure *S. cerevisiae* without traces of other yeast species. The same positive result but with 100 % of *Torulaspora delbrueckii* (OTU 3) can be reported for the applied sample of TUM T 90. TUM T 90 is an alternative brewing yeast of the species *T. delbrueckii*, which was recently used for fermentation of novel beers [20].

As presented in figures 1 and 2, the reads of samples TUM 184 and TUM 68, showed 94.29 % respectively 96.5 % of *S. cerevisiae* OTU 1, but also 2.94 % respectively 0.61 % of *Schizosaccharomyces pombe* (OTU 7), 2.41 % respectively 0.34 % of *Hanseniaspora uvarum* (OTU 4), and 0.35 % respectively 2.37 % of *Torulaspora delbrueckii* (OTU 3). And in the case of TUM 184, 0.2 % of the reads corresponded to the class *Saccharomycetaceae* (OTU 18); whereas for TUM 68, 0.18 % of the reads corresponded to uncultured yeast (OTU 28).

Similar results as for TUM 184 and TUM 68 were detected with varying species for the supposed pure samples of TUM 127, TUM SL17, TUM 523, TUM 536, TUM Bretta 1 and TUM V1 visible in table 3.

These results can indicate different possible incidents with varying causes. Firstly, the culture may not have been as pure as thought prior to investigation. This could be due to contaminations of the pure cultures by other species. As the samples were not prepared from single colonies, it is likely that they were pure cultures of one strain of one species. The fact that more than one species was detected by HTS could potentially indicated the presence of syntrophic cultures. Syntrophic cultures are known to be very difficult to separate, as cells of different sizes adhere together [21]. The detected species are known to be able to grow in wort and might therefore be able to survive in close proximity with each other, making it hard to separate them on agar by traditional separation techniques. As *H. uvarum* represents a very fast-growing yeast species (generation time <1 h at 30 °C) [22], much faster than *Saccharomyces cerevisiae* strains (generation time <3 h at 30 °C), a prior negative detection of this contamination is very unlikely but cannot be definitively excluded. Other possible reasons are genetic changes on the ribosomal DNA of the *Saccharomyces* strains by differing impacts [23; 24]. One potential cause might be horizontal gene transfer as reported before by Xie et al. [25]. Another reason can be hybridization between the differing detected species [24]. As the 26S rDNA D1/D2 region is found in amounts of 100-200 tandem repeats on chromosome XII of many yeast species [26; 27], hybridization or horizontal gene transfer could lead to varying sequences during the tandem repeats [24; 25]. These variations may potentially be identified by whole genome sequencing which will be the task in further research on this topic.

The total reads of the fragments in the sample of strain TUM 193

Table 3 Distribution of species results of the OTUs detected for six supposedly pure yeast culture samples

Strain	Supposed pure species	OUT ID	According species	Number of reads	Percentage of total reads [%]
TUM 127	*S. cerevisiae*	1	*S. cerevisiae*	5563	99.49
		4	*H. uvarum*	19	0.33
		7	*S pombe*	9	0.16
TUM SL 17	*Saccharomycodes ludwigii*	6	*S. ludwigii*	5146	97.66
		7	*S. cerevisiae*	123	2.44
TUM 523	*H. uvarum*	4	*H. uvarum*	7017	99.84
		1	*S. cerevisiae*	11	0.16
TUM 536	*S. pombe*	7	*S. pombe*	2140	73.56
		4	*H. uvarum*	769	26.43
TUM Bretta 1	*Brettanomyces bruxellensis*	11	*Dekkera anomala*	1189	54.51
		1	*S. cerevisiae*	599	27.46
		6	*Saccharomycodes ludwigii*	299	13.7
		99	*B. anomalus*	53	2.4
		3	*T. delbrueckii*	30	1.37
		4	*H. uvarum*	11	0.50
TUM V1	*S. cerevisiae*	1	*S. cerevisiae*	5779	87.45
		3	*T. delbrueckii*	668	10.10
		26	*Uncultured yeast*	68	1.02
		28	*Uncultured yeast*	56	0.84
		7	*S. pombe*	25	0.37
		4	*H. uvarum*	12	0.18

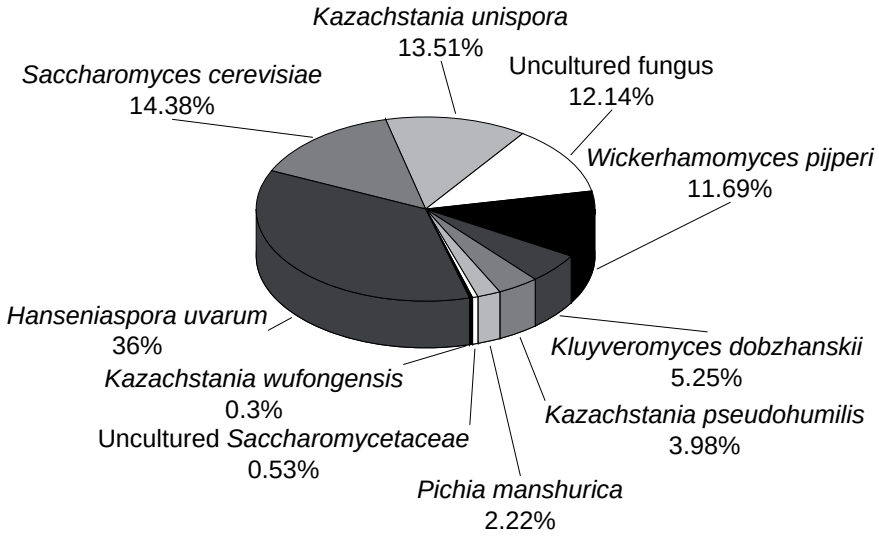

Fig. 3 Composition of OTUs displayed in percent, detected for the pooled sample (percentages result from total of 6675 reads).

showed 100 % of OTU 1 (*S. cerevisiae*). As this yeast strain is a bottom fermenting yeast of the species *S. pastorianus*, which is a hybrid of *S. cerevisiae* and *S. eubayanus* [28], it is concluded that the discovered OTU contained low or no amounts of the rDNA coming from *S. eubayanus* on this particular region. A small difference between these two species on the 26S rDNA D1/D2 region is possibly the case. When this OTU was BLASTed against *S. eubayanus* 26S rDNA D1/D2 it showed a similarity of 99 %. As mentioned above the use of the 26S rDNA D1/D2 region was only a first proof of principle. It will be followed by further investigations of the ITS1 region, which might be more discriminative and, in this case, more usable than the 26S rDNA D1/D2 region.

Figure 3 displays the results of the OTU distribution of the pooled sample. This sample was produced to create a mixture of unknown and known species. The sample contained inoculated strains of the species *S. cerevisiae*, *Kazachstania unispora* and *Hanseniaspora uvarum*. Further unknown interfering yeast species from the environment were present in the sample as it was spontaneously fermented by exposing the cold wort to the environment for one day. It is apparent that more than the inoculated species were detected (*K. pseudohumilis*, *Wickerhamomyces pijperi*, *Pichia manshurica*, *K. wufongenensis*) (Fig. 3). The inoculated species were reliably detected. The actual proof of detecting the pooled species was successful as visible in figure 3.

4 Conclusion/Summary

Defined strain starters (DSS) are one of the main factors contributing to the success of the fermentation industry. Most of the products created from fermentations are of high pureness and exceptional quality. The high quality is due to a predictable, efficient, controllable and reproducible fermentation performed with a defined starter [1]. Quality control of the purity of the starters is critical to the aussurance of high quality fermentation products. Over the last century, quality control has improved in sensitivity, speed, and reliability. New methods have always been the key to even higher quality. Over the last decades, molecular biological methods like Real-Time PCR and DNA-Fingerprinting have accelerated the improvements in quality control. HTS has proven to provide a major step forwards concerning detection of multiple species of yeast in one sample with unknown composition. Here, we demonstrated that the purity of yeast starters for beer, wine and special beers can be assured by HTS. As this was just a proof of principle, further adjustments to this method have to be performed in order to reliably detect interfering yeast species. Some results did not show the expected outcome, hinting at potential genetic variation in some yeast strains. As it cannot be completely excluded that the supposedly pure cultures had a certain amount of interfering yeasts, this will be analyzed in a further study. Despite these findings, the results indicate a new promising tool for non-targeted quality control. To improve further, other genetically diverse regions, such as internal transcribed spacers (e.g. ITS1) will be applied, as they promise to be more discriminative than the 26S rDNA D1/D region [29]. Furthermore, as the results of BLASTing against a public database in the present study have highlighted, a reference library for these specific regions is still needed in order for reliable identification at the species level to be fully achieved.

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