Selection and identification of thermophilic yeast strains in leachate from the organic waste heap in Phu Luong district, Thai Nguyen province, Vietnam

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Abstract. The study was carried out to isolate and select useful thermophilic yeast strains in the process of organic domestic waste treatment in Phu Luong - Thai Nguyen. Research results from 23 samples of rust have isolated 10 strains of yeast on YPG medium at 40 oC. Among them, 6 strains of yeast were selected with the ability to grow and develop in a wide temperature range from 20-45 oC. The results of identification combined with morphological, physiological, and biochemical characteristics of yeast strains showed that, out of 6 selected strains, there were 3 strains belonging to the genus Saccharomyces (Saccharomyces sp. TNY13.01, Saccharomyces sp. TNY22.01), Saccharomyces cerevisiae TNY13.09), 2 strains of the genus Candida (Candida sp. TNY23.01, Candida tropicalis TNY23.126) and 1 strain of the genus Papiliotrema (Papiliotrema laurentii TNY23.127). Among them, the identified strain Saccharomyces cerevisiae TNY13.09 has the ability to grow at 45, tolerates a wide pH range of 4.0–8.5, has a positive catalase reaction, is capable of using a variety of carbon sources, and belongs to class I biosafety group. On that basis, Saccharomyces cerevisiae TNY13.09 has the potential to be further researched and applied as additional microbial inoculants to the organic waste heap.

1 Introduction

Yeast has a very important role in life and is present in the fermentation process of food processing [1]. In organic waste treatment, yeast has also been shown to play an important role in the fermentation process that breaks down organic waste [1, 3]. Treatment of organic waste by microbial fermentation is a new technology with many outstanding advantages compared to other methods such as landfilling and incineration. Biological treatment of organic waste includes the study and isolation of microorganisms capable of decomposing organic compounds in domestic waste, in order to create products that promote the process of organic waste growing rapidly. The process of decomposing and deodorizing turns organic waste into safe organic fertilizers for agricultural production. This problem has now been

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studied and applied in many places. However, the majority of studies focus on bacterial and actinomycete strains with little mention of yeast strains [4, 5]. In the early stages of decomposition, organic acids are formed, creating a compost environment with a pH between 5.5 and 8.5 which is optimal for the activity of composting microorganisms. Groups of filamentous fungi, actinomycetes, and bacteria grow and break down lignin and cellulose. However, in the middle of the composting process, when the temperature of the compost pile is high, the system also becomes anaerobic and the accumulation of acids can lower the pH to 4.5. This is an adverse condition that severely limits the activity of beneficial microorganisms involved in the composting process. Under acidic conditions, anaerobic organic matter can accumulate rather than decompose. Sundberg (2021) has shown that organic acids formed are associated with odor problems in food waste composting plants (occurring at low pH stages). At this stage, yeast strains that are able to adapt to low pH, and temperature tolerance will actively grow and metabolize organic acids, neutralize the pH of the compost, and create favorable conditions for microorganisms. Beneficial organisms continue to grow and break down organic compounds, thereby helping to reduce the risk of odor emissions [4, 5, 6]. Moreover, because the waste composition and environmental conditions are very different in each place, the isolation and selection of indigenous yeast strains that can withstand high temperatures have high practical significance in the application of inoculants. effective microorganisms for each area [7, 8].

Phu Luong is a mountainous district located in the northern region of Thai Nguyen province with a population of over 100,000 people (ethnic minorities account for 50%), and the main agricultural economy (Phu Luong District People's Committee, 2022). With the peculiarity of being a mountainous agricultural district with many difficulties, promoting propaganda and implementation of waste classification, making use of organic waste as fertilizer for crops has high practical significance. Moreover, treating waste with microbial inoculants with indigenous microorganisms helps to improve the efficiency of local waste treatment [9]. This study was initially conducted to select a thermophilic yeast strain in leachate from an organic waste heap in the PhuLuong district, Thai Nguyen province, Vietnam to add to the set of helpful indigenous microorganisms, in the production of organic waste treatment inoculants.

2 Materials and methods

2.1 Materials

A total of 23 leachate samples were collected between September 2022 and January 2023 from the organic waste compost pile in PhuLuong district, ThaiNguyen province, Vietnam. Organisms should be handled under appropriate health and safety conditions in the laboratory. Isolation strains were preserved in 20% glycerol (v/v) at -80oC at the Laboratory of Biotechnology Sub-Institute of Joint Vietnam - Russia Tropical Science and Technology Research Center.

2.2 Methods

2.2.1 Method of collecting leachate samples

Wastewater at the general landfill of Phu Luong district (a landfill in which it is being treated) flows into areas and wastes. Wastewater samples were collected by pouring a 50mL falcon tube into a dynamic puddle, 5 cm deep, allowing water to leak into the tube [9].

Sample treatment: After being transported, the leachate sample was transported to the laboratory of the Biotechnology Sub-Institute/Vietnam - Russia Tropical Center for 24 hours to conduct further studies.

2.2.2 Isolation and identification of yeast strain profiles

From the collected leachate sample, take 10mL of leachate sample, add 90ml of YPG (Yeast extract-Peptone-Glucose medium), and incubate in a sterile conical flask at 40oC for 24 hours to isolate thermophilic yeast. Yeast strains were isolated on YPG medium supplemented with chloramphenicol at 30 mg/mL. Preliminary identification of yeasts isolated by morphological methods based on the yeast taxonomy key of Kurtzman and Fell [10].

+ Test for the heat resistance of yeast strains: Put the isolated yeast strains on a petri dish containing YPG agar. Incubate petri dishes at different temperatures: 20-25-30-35-40-45-50^{o}C for 48 hours. Observe the formation of the colony with particular characteristics (similar to when growing at 30^{o}C) of yeast strains on agar. Selection of yeast strains capable of growing at temperatures of 45^{o}C [4, 11].

+ Determination of morphological and biochemical characteristics of selected thermophilic yeast strains, including determination of the ability to ferment glucose, fructose, maltose, lactose, sucrose, trehalose, raffinose, and galactose [10, 13]. The yeast strains were then cultured to the same level of bile in YPG solution supplemented with 1% of different carbon sources. The yeasts were grown at 150 rpm for 24 hours at an adaptive temperature of 30oC, and the optical density was measured at OD540 nm. An increase in optical density in each culture vessel was noted as evidence of yeast growth and their tolerance to heat (Tahía et al., 1983). Cell/spore morphology was observed using an Axio Image 2 micro microscope (Imager.Z2) with an integrated Zeiss Axiocam 503 Color Camera Kit.

+ Tests confirming the production of extracellular hydrolytic enzymes were conducted using the agar plate diffusion method [7, 12]. The isolates were cultured on substrate agar plates (1%, w/v, cellulose, gelatin, starch, and tween 80) to determine cellulase, protease, amylase, and lipase activities. Plates were incubated for 48 hours at 35°C and enzyme activity was detected.

2.2.3 Identification by sequencing method

Cloning target genes by PCR technique from total DNA: Total DNA of fungi was extracted using a Plant DNA Isolation Kit (Norgenbiotek, Canada). Total DNA was checked by electrophoresis on 1% Agarose gel, using 1X TAE buffer, stained with RedsafeTM Nucleic Acid gel Stain, and performed on Biorab electrophoresis equipment. The selected yeast strain was partially sequenced by PCR using primers ITS1 (5'TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Korabecna, 2007). Each PCR reaction has a volume of 25 µL with ingredients: 7 µL H2O deionized water, 12.5 µL PCR Master mix kit (2X), 1.25 µL forward primer (10 pmol/µL), 1.25 µL primer reverse (10 pmol/µL), 3 µL DNA (10 - 20 ng). The reaction was performed on a model GeneAmp™ PCR System 9700 (Life Technologies Applied Biosystems, Singapore). The thermal cycle of the PCR reaction includes 94^{o}C for 3 minutes; followed by 35 cycles in series with steps: 94^{o}C for 45 seconds, 55^{o}C for 45 seconds, and 72^{o}C for 45 seconds; end the multiplication reaction at 72^{o}C for 10 min, keeping the product at 4^{o}C [8, 12].

Sequencing and sequence correction: PCR products were electrophoresed on 1.5% agarose gel and nucleotide sequencing was performed at NamKhoa Biotek company, Vietnam. The DNA sequences after sequencing were corrected and removed for noise with the help of ChromasPro2.1.6 software (Technelysium, 2013) and compared with those already on Genbank (using the BLAST tool) in NCBI - http:www.ncbi.nlm.nih.gov/BLAST).

Analytical sequences were aligned using Bioedit software v7.0.5.2 (Hall, 1999). Areas that cannot be sorted are removed prior to analysis. The experiments were repeated 3 times, the research data were statistically processed using Excel 2010 software.

3 Results and discussions

3.1. Results of isolation of indigenous yeast strains from organic landfill leachate

To enhance the ability to process a variety of sources of organic compounds from domestic waste, and to help reduce the stench generated during the composting process, the task proposed to select more thermophilic yeast strains. The leachate samples collected from the organic waste compost pile in PhuLuong district, ThaiNguyen province, Vietnam were used to isolate and select thermophilic yeast strains on YPG agar with chloramphenicol antibiotic with a concentration of 30 mg/mL at 40ºC. After 48 hours of incubation, a variety of yeast was visible on most plates. Purification of morphologically distinct colonies led to 12 single cultures.

The application of different incubation temperatures resulted in diverse fungal colonies with different growth rates. The results obtained 12 strains of yeast continued to be tested for their ability to grow at temperature conditions from 20 - 50ºC (Table 1). The results show that plates incubated at 25-40°C showed quick yeast strains growth. Incubations at 45 °C displayed no overgrowth, and a reduced number of fungal colonies colonized plates. Different yeast strains were obtained from each incubation temperature. Six isolated strains, TNY13.01, TNY13.09, TNY22.01, TNY23.01, TNY23.126, and TNY23.127 are the only strains obtained from both 20°C and 45°C, suitable for the early stage of the composting process should be selected for the study.

Table 1. Growth ability of selected yeast strains at different temperatures

Isolate code	Growth ability						
	20ºC	**25ºC**	**30ºC**	**35ºC**	**40ºC**	**45ºC**	**50ºC**
TNY03.16	+	+	++	++	+	-	-
TNY06.11	-	+	++	+	+	-	-
TNY11.07	-	+	++	+	+	-	-
TNY13.01	+	+	+++	++	++	+	-
TNY13.09	+	++	+++	++	++	+	-
TNY15.07	-	++	++	++	+	-	-
TNY17.12	-	+	++	++	+	-	-
TNY18.17	-	+	++	++	+	-	-
TNY22.01	+	+	++	++	+	+	-
TNY23.01	+	+	++	++	+	+	-
TNY23.126	+	+	++	++	+	+	-
TNY23.127	+	+	++	+	+	+	-

3.2 Morphological, biochemical, physiological characterization and extracellular enzyme activities of yeast strains

From leachate samples, six strains of yeast were isolated and selected. The morphological, biochemical, and physiological characteristics and enzyme activity of these 6 yeast strains are described in Table 2, Table 3, and Fig.1.

Table 2. Characteristics of yeast strains isolated from leachate

Isolate code	Morphological characteristics (on YPG agar plate/ 48h)		Biochemical and physiological characteristics		Enzyme activity				
	Colony morphology	Cell shape	Gr. temp range, ^oC	Gr. pH range	Catalase	Amylase	Protease	Lipase	Cellulase
TNY13.01	Round shape creamy white, dry surface smooth, smooth edges, colony size 2.1-3.5 mm	Oval shape, multipolar budding	20–45	4.5-8.0	+	+	+	+	-
TNY13.09	Round shape, clear white, dry surface smooth, size 1.5-2.5 mm	Oval shape, bipolar budding	20–45	4.0–8.5	+	+	+	+	+
TNY22.01	Round shape, milky white, smooth dry surface, serrated cover, size 2.5-3.5 mm	Oval shape, multipolar budding	20–45	4.0-8.0	+	+	+	+	+
TNY23.01	Round shape, milky white, smooth dry surface, serrated cover, size 2-3 mm	Round/oval shape	20–45	4.5-7.5	+	+	+	-	-
TNY23.126	Round shape, clear white, smooth surface, smooth edges, size 2.0-2.5 mm	Round shape	20–45	4.5-7.0	+	+	+	-	-
TNY23.127	Round shape, milky white, smooth dry surface, size 3.0-3.5 mm	Round shape	20–45	4.5-6.5	+	+	+	-	+

Notes: +: positive; -: negative

Table 3. The assimilation of primary carbon sources by yeast strains

Isolate code	Glucose	Fructose	Maltose	Sucrose	Lactose	Galactose	Trehalose	Raffinose
TNY13.01	+	+	+	+	-	+	-	
TNY13.09	+	+	+	+	-	+	-	
TNY22.01	+	+	+	+	-	+	-	
TNY23.01	+	+	+	+	-	-	+	-
TNY23.126	+	+	+	+	-	-	+	-
TNY23.127	+	-	+	+	+	+	+	+

Notes: +: positive; -: negative

The isolates were cultured at 7 temperatures (from 20°C to 50°C). Nearly 6/12 yeast strains could grow at 20°C -45°C and usually grew optimally at 30°C or 35°C (Table 2). To estimate the yeast's ability to utilize nutrients in the natural environment, they were initially characterized to produce 4 extracellular enzyme activities (amylase, protease, lipase, and cellulase). As shown in Table 2, all yeasts exhibited at least three-quarters of enzyme activity, further enhancing their biotechnological/industrial exploitation potential.

It was observed that yeast cells develop different growth and morphological characteristics when grown on different carbon sources. The effects of different carbon sources on the growth of yeast strains were investigated [10, 13]. The carbon sources used include sugars such as glucose, galactose, fructose, and sorbitol, organic acids lactate and pyruvate, the fatty acid oleic acid, and mixed amino acids. The effects of different carbon sources on yeast strains was shown in table 3. After the growth of the cells was monitored for 48 hours, it was shown in table 3 that the cells grown in sugars such as glucose, fructose, and galactose had a better growth rate as compared to the cells grown in the other carbon sources used in this study.

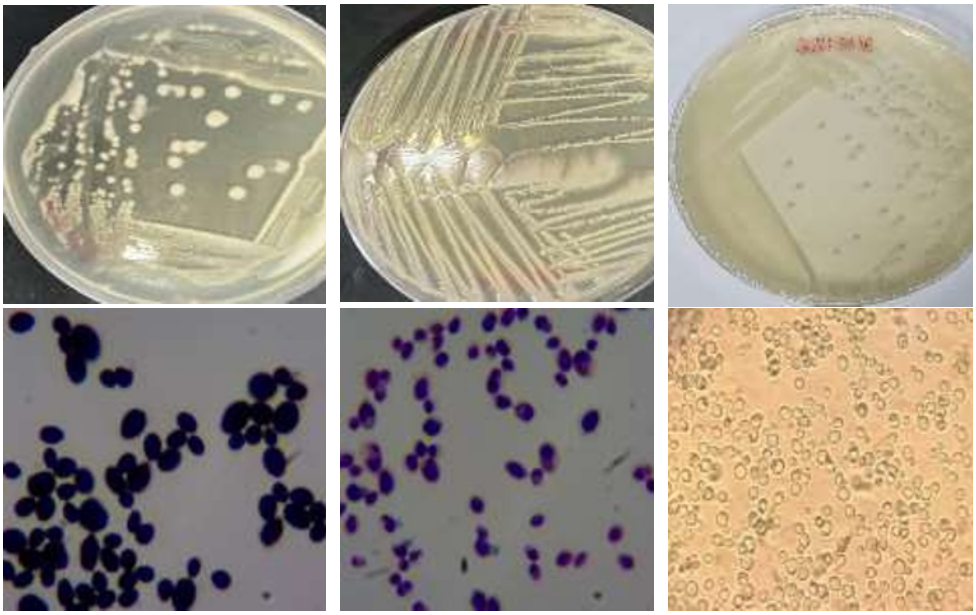

Fig. 1. Colony and cell morphology of some isolated yeast strains

3.3 ITS-rDNA gene sequencing results and identification of 6 yeast strains

The total DNA of 6 yeast strains was successfully isolated with high DNA quality. The OD measurement results show that the OD260/OD280 index of the samples is between 1.8 and 2.0 (an indicator indicating high-purity DNA content). We purified total DNA using a DNA extraction kit (Genomic Purification Kit) to ensure accurate sequencing results.

The target gene region ITS-rDNA was successfully amplified using primers ITS1/ITS4 with an annealing temperature of 55oC for the study sample. The PCR product is about 600 bp in size for the ITS-rDNA gene region. The quality of the PCR product is demonstrated when electrophoresis on 1.5% agarose gel has only a single band, bright and dark, which is qualified for nuclear gene fragmentation to decode nucleotide sequences.

PCR products after being purified are used as template DNA for PCR reactions to reading nucleotide sequences. The sequencing reaction was performed in two directions by the direct sequencing method. The results of sequencing the ITS-rDNA gene region give an electrophoretic image with clear, intense, and clear fluorescence peaks (Appendix). After removing primer sequences and noisy signal regions using ChromasPro 2.1.6 software, we obtained nucleotide sequences (approximately 585 nucleotides in length) of yeast strains.

Sequences obtained from yeast strains were checked for similarity with sequences available on Genbank using the BLAST tool. The search results showing the highest similarity sequences are shown in that the molecular identification of the isolates based on barcode sequences resulted in a taxonomic assignment on species level for 2 isolates. The identification of 4 isolates was only possible on the genus level (Saccharomyces sp., Candida sp., and Papiliotrema sp.) due to low similarity with reference material (Table 4), which might, therefore, represent novel-undescribed species. Yeast is an important part of the microbiota observed during composting because of their ability to decompose dry recalcitrant acid and low-nitrogen-containing substrates in comparison to bacteria [3, 5]. As many studies have shown the evolution of the fungal community in the process of composting, Saccharomycetales yeast is associated with the early stages of composting [4]. In the initial composting stage, Saccharomycetales was the dominant order comprising the total population. Besides Saccharomycetales, Candida species are often found in composts [6]. Candida sake is reported to be a human health pathogen associated with endocarditis [1]. Over time, the abundance of Saccharomycetales decreased and was equal to 4% in the final product [9].

Table 4. Identification of six yeast strains based on ITS-rDNA sequences

Isolate code	Identification	Accession number/ NCBI	Similarity with reference material
TNY13.01	*Saccharomyces cerevisiae* IAL7408	OL848036.1	99.67%
TNY13.09	*Saccharomyces cerevisiae* LY47	OQ519837.1	100%
TNY22.01	*Saccharomyces cerevisiae* IAL7408	KM589492.1	99.67%
TNY23.01	*Candida tropicalis* MYA-3404	MH545915.1	99.45%
OQ677532	*Candida tropicalis* CBS 1920	MK394119.1	100%
OQ677533	*Papiliotrema laurentii* IIF4SW-F2	KY218691.1	99.72%

Table 5. The names and biosafety of selected microorganisms

Isolate code	Accession number/ NCBI	Identity	Biosafety (Group)
TNY13.01	OQ677530	Saccharomyces sp. TNY13.01	I
TNY13.09	OQ677528	Saccharomyces cerevisiae TNY13.09	I
TNY22.01	OQ677529	Saccharomyces sp. TNY22.01	I
TNY23.01	OQ677531	Candida sp. TNY23.01	II
OQ677532	TNY23.126	Candida tropicalis TNY23.126	II
OQ677533	TNY23.127	Papiliotrema laurentii TNY23.127	II

However, national and local policies for categorizing and containing biological agents should be followed. Organisms must be handled under appropriate health and safety conditions in the laboratory following a risk assessment. The above strains were compared with the European Community's List of Safe Microorganisms and the European Community's List of Restricted Microbial Species and Biosafety in Microbiological and Biomedical Laboratories (Centers for Disease Control and NIH), which revealed three strains belonging to the genus Saccharomyces (TNY13. 01, TNY22.01, and TNY13.09) are in the biosafety level I group and not in the restricted microbiological group [14]. Two strains of Candida species (TNY23.01, TNY23.126) and one strain of Papiliotrema (TNY23.127) both achieved biosafety level II group which is at risk of infecting humans (Table 5). Even so, it is less likely to spread to the community and usually has effective prevention or treatment measures, and is not a restricted-use microorganism. Among these strains, the yeast strain Saccharomyces cerevisiae TNY13.09 has the ability to grow widely in temperature conditions, temperature tolerance up to 45oC, wide pH range from 4-8.5, the ability to use diverse carbon sources, positive catalase....so there are many prospects to be added to microbial inoculants to treat diseases from organic waste.

4 Conclusions

A total of 12 thermophilic yeast strains were isolated from 23 leachate samples at 40oC. There are six strains of yeast that can grow at temperatures from 20 to 45oC. Most yeasts are capable of assimilating different carbon sources (consumption of 4 to 6 of the 6 tested carbon sources). All species exhibited at least 2 of the 4 extracellular enzymatic activities tested (protease, amylase, lipase, and cellulase). Among them, lipase, and amylase activities predominated, while protease and cellulase were less common. Molecular analysis based on rDNA sequence revealed that 6 yeast strains belong to 3 genera, of which 3 yeast strains belong to Saccharomyces genus (TNY13. 01, TNY22.01, and TNY13.09), 2 yeast strains belong to the genus Candida (TNY23.01, and TNY23.126) and 1 yeast strain belongs to the genus Papiliotrema (TNY23.127). Which strains of Saccharomyces (TNY13. 01, TNY22.01, and TNY13.09) belong to the biosafety level 1 group, so they were selected for additional research into microbial inoculants for organic waste treatment.

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References

1. C. Somdatta, et al., Int. J. of Chem. Sc., **9** (2), (2011)
2. O. R. Barrera, et al., Italian J. of Animal Sc., **17** (4), 1054-1058 (2018) DOI: 10.1080/1828051X.2018.1448724
3. F. Witfeld, et al., Mycol Progress, **20**, 325–339 (2021). https://doi.org/10.1007/s11557-021-01674-z
4. K. Nakasaki, and H. Hirai, Waste Management, **65**, 29–36 (2017) doi:10.1016/j.wasman.2017.04.019
5. C. Sundberg, et al., Biores. Technol., **95** (2), 145–150, (2004). doi:10.1016/j.biortech.2004.01.01
6. T. Freitas, et al., Advances in Microbiol. **04**, 796-807 (2014)
7. M. Carrasco, et al., BMC Microbiol, **12**, 251 (2012). https://doi.org/10.1186/1471-2180-12-251

8. J.K. Carvalho, et al., Biotechnol Rep (Amst), **30**, e00630 (2021); doi: 10.1016/j.btre.2021.e00630.
9. C.S. Kischinevzky, et al., Microorganisms, **10**, 794 (2022) https://doi.org/10.3390/microorganisms10040794
10. C.P. Kurtzman, J.W. Fell, Teun Boekhout, *The Yeasts - A Taxonomic Study, 5th Edition*, (eBook, ISBN: 9780080931272, 2011)
11. E. Leithy, et al., J. of Productivity and Development, **12**, 153-171 (2007)
12. R. Bussamara, et al., Bioresource Technology, **101** (1), 268–275 (2010). doi:10.1016/j.biortech.2008.10.06
13. B. Lok, et al., Med Mycol., **59** (2), 115-125 (2021). doi: 10.1093/mmy/myaa080. PMID: 32944760.
14. *Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th edition*, (Centers for Disease Control (CDC) and NIH, 2019)