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Different Methods for DNA Extraction from Yeast-*Candida famata* Isolated from Toddy

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Authors' contributions

This work was carried out in collaboration of all authors. Author TS did the extraction procedure of DNA. Author SKG designed the biochemical characterization, managed the analysis of whole study and wrote the manuscript, and author AC managed the literature search. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Isolation and biochemical characterization of yeasts from toddy and standardization of best method for DNA extraction from yeast.
Study Design: Biochemical characterization of yeast and genomic DNA extraction by manual and kits methods.
Place and Duration of Study: Department of Microbiology, Institute of Genetic Engineering, Badu, kol-128, India and Molecular Mycopathology Lab, P. G. Department of Botany, Ramakrishna Mission Vivekananda Centenary College, Rahara, kol-118, India, from November 2012 –April,2013.
Methodology: Toddy was collected in sterilized polythene bags from palm tree (*Borassus flabellifer* L*;* Family: *Arecacea*) in the morning, from Badu, 24-parganas (N) India. Isolation of yeasts was done by the method of Beech and Davenport [15] using MA (Malt extract) medium. Biochemical Identification was performed by using basal medium and procedure [1,2,15]. Genomic DNA extraction was done by manual and kits methods (Uniflex^{TM} DNA isolation Kit). Quality of extracted DNA was checked by the absorbance ratio (A_{260} / A_{280}) ranged from 1.8 to 2.0.
Results: By performing morphological, microscopical and biochemical characterization

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the isolated yeast from toddy was identified as *Candida famata* consulting with the key of yeast published [1,2].
The Uniflex™ DNA isolation Kit method is much more convenient way to get pure and high quality DNA than the manual methods.
Conclusion: Isolated yeast from toddy was identified as *Candida famata.* The genomic DNA of *Candida famata* was extracted purely by Uniflex™ DNA isolation Kit. This method was better and more convenient than manual method.

Keywords: Toddy; Yeast; biochemical characterization; DNA extraction; Kit method.

1. INTRODUCTION

Yeasts are unicellular fungi. They may be Ascomyceteous, Basidiomyceteous or Deuteromyceteous fungi. Some yeasts are filamentous in specific environmental conditions. They are called dimorphic yeasts. Yeasts are cosmopolitan. They are generally present in natural sources such as leaf surface, fruit surface, soil, flowers, various juices (toddy juice, date palm juice), molasses, animal surfaces, etc [1]. They are generally saprophytic but some are also pathogenic to humans. Therefore, their diversity in natural sources is very immense. According to Kurtzman and Fell [2], there are about 100 genera and 700 species of yeasts. In most advanced countries like USA, Japan and Germany systematic study of yeast flora from natural sources has taken much emphasis in biocontrol of pathogens. Although, India including West Bengal has vast natural sources for yeast, its study of yeast flora is limited.The post harvest rots of fruits and vegetables are chronic problem in post harvest technology i.e fruit industry. The major post harvest rot of orange (*Citrus reticulate* Blanco) is caused by *Penicillium digitatum or P. italicum.* Potential use of yeasts as biocontrol agents of soil born fungal plant pathogens and as plant growth promoters were recent investigated by EL- Tarabily and Sivasiyhamparam [3] and have been used extensively for biological control of post harvest diseases of fruits & vegetables[4].The yeast *Torulapsis candida (Candida famata*) effectively controls *Penicillium digitatum* infection on *Citrus* fruits [5].Wild variety of yeasts have Can*dida saitoana and C. oleophila* control post harvest diseases of apple and *Citrus* fruits[6].*Candida famata* gave maximum percent of radial inhibition of growth (70.24 PIRG)followed by *Pichia membranifaciens* (68.21 PIRG), *Rhodotorula mucilaginosa* (60.56 PIRG)[7]. *Candida famata* (also known as *Debaryomyces hansenii* and *Torulopsis candida*) is a commensal yeast found in cheese, dairy products and the environment. *C. famata* is now an emmerging human pathogen and it accounts for 0.2%–2% of invasive candidiasis of human[8].The cell wall is the main obstacle for quick and easy lysis of yeasts and therefore it must be disrupted for efficient recovery of genomic DNA (gDNA). Conventional methods for gDNA preparation from yeast cells utilize either enzymatic degradation generally followed by lysis of cells with detergent and extraction of gDNA with phenol-chloroform. When analyzing large number of samples these methods are time consuming and relatively expensive procedure for extracting DNA. Various methodology have been reported for DNA extraction [9,10,11]. Two simple and easy protocols for extracting the high quality DNA from yeast have been employed in this work. One protocol is done by preparing the lysis buffer, organic solvent and dissolving buffer manually and the second was the specific kit (Uniflex™ DNA isolation Kit) method. Pure DNA extraction is very necessary for routine genotyping of yeasts either by simple detection of PCR products or RAPD(Random Amplified Polymorphic DNA), or for initial amplification of genomic DNA for sequencing; procedures that are widely used for analysis of scientific, environmental, industrial and clinical samples.

The main objective of this study was to screen the best method of these two procedures to extract pure and high quality DNA of C.famata and getting the convenient, easy, less time consuming method for the yield of high quality and pure DNA having the absorbance ratio (A_{260} / A_{280}) ranged from 1.8 to 2.0.

2. MATERIAL AND METHODS

2.1 Isolation and Purification Procedure of yeast

Toddy was collected in sterilized polythene bags from palm tree (*Borassus flabellifer* L;Family: *Arecaceae*) in the morning, from Badu, 24-parganas (N) India and brought in laboratory. One ml of toddy was serially diluted in sterile distilled water upto 10^{-4} and these were plated on sterile MA medium (MA; Malt extract, 2g;Agar,2g; Distilled water,100ml in sterile petridish and incubated for 48 hours at 28 ± 2ºC in BOD incubator [2,12].

Single cell of the isolate was obtained by streaking loop full of cells on MA medium and transferring well isolated colonies to MA. The isolates were maintained on Malt-Yeast – Glucose- Peptone –Agar medium (Dry malt extract, 3g;dry yeast ,3g; peptone,5g; D-glucose, 10g; agar,20g; distilled water, 1L) at 28±2ºC with monthly subculturing.

2.2 Identification of Yeast

Identification of isolated yeast up to species level was carried out on the basis of standard morphological, and physiological /biochemical tests [1,2,13,14].

2.2.1 Morphological and Microscopical investigation

The colonies were observed and described on MA and MYGPA medium. The isolated yeast was also grown in MA and MYPG broth for determination of their cultural characteristics (pellicle, sedimentation or ring formation). In certain cases, the isolate was grown on sterile slices of carrot for induction of ascospore formation.

The isolated yeast grown on MYPG broth and its slide was prepared and stained by crystal violate solution and observed under oil emersion lens (100xX10x) of compound light microscope.

2.2.2 Biochemical characterization

For carbon and nitrogen assimilation, the basal medium [1,15] was used and the results were determined after the 3th, 7th, 14th, 21th and 28th day.

The ability of some carbohydrates for anaerobic assimilation (fermentation) was determined by using Durhan glass tubes after 3 weeks. The quantity of the tested carbohydrates was 2%. For Diazonium blue –B (DBB) test, a ten day old culture on MYPGA was held at 55°C for three hours and then flooded with ice –cold DBB reagent. The reagent was prepared by dissolving diazonium blue salt (Sigma) in cold 0.5M-tris –HCL buffer pH 7.0 at 1mg /ml.The reagent was kept ice –cold and used within few minutes of preparation. Other additional tests such as starch formation, urea hydrolysis, cyclohexamide (0.01% or 0.1%), were performed [1].

2.3 DNA Extraction Method

2.3.1 Extraction of DNA from yeast culture by preparing the buffer in laboratory

Overnight grown (at 30ºC) yeast culture ($1x10^7$cells /ml) was taken in an centrifuge tube. Yeast culture was centrifuged for 5 min at 1500 rpm and it was resuspended in 0.5 ml of sterile distilled water. Cells were transferred to eppendorf and it was spin down for 5 sec. at 14,000 rpm. Supernatant was discarded and pellet in the residual water was vortexed 200 µl of yeast lysis buffer (Triton X -100, 10% SDS, 5 M NaCl, 0.5 MEDTA, 1M Tris and distilled water), 200 µl of organic solvent (phenol: chloroform : isoamyl alcohol in the ratio of 25:24:1), and 0.3g of glass beads were added. It was again vortexed and 200 µl TE was added. It was centrifuged again and aquous phase was transferred to a new tube and 1 ml of ethanol was added. Supernatant was discarded after centrifugation and 400 µl TE (Tris-EDTA) and 4 µl RNase was added to the pellet and kept at 37ºC. 10 µl of 4M ammonium acetate and 1ml ethanol was added. Again it was spin and supernatant was discarded. It was washed with 70% ethyl alcohol, then pellet was air dried and resuspended in 50 µl TE. Agarose gel (1%) electrophoresis was done and band was observed under UV transluminator. Purity was checked by taking the absorbance ratio in spectrophotometer. This was done according to the published keys [9,10,11].

2.3.2 Uniflex™ DNA isolation Kit method

Overnight grown (at 30ºC) yeast culture ($1x10^7$cells /ml) was taken in an centrifuge tube. Yeast culture was centrifuged for 5 mins at 6000 rpm. 1 ml of uniflex™ buffer 1 and 10 µl of RNase A were added to the pellet , mixed well and kept for 30 mins at 37ºC for incubation. 1 ml of 1:1 phenol: chloroform was mixed to the lysed cell. It was centrifuge at 10,000 rpm for 15 mins at rt [room temperature]. The aquous layer was transfered to a fresh tube and uniflex™ buffer 2 was added and mixed well. It was again centrifuge at 12,000 rpm for 15 mins and supernatant was discarded. 70% ethanol was added to the pellet and centrifuged at 10,000 rpm for 10 mins and supernatant was discarded. The pellet was air dried and resuspended in 50 µl UNIFLEX™ elution buffer and it was stored at -20ºC. Agarose (1%) gel electrophoresis was done and band was observed under UV transluminator. Purity was checked by taking the absorbance ratio in spectrophotometer.

3. RESULTS AND DISCUSSION

The data presented in the Table 1 showed that the isolated yeast colony morphology was white and round; its margin was undulating but elevation was convex. The microscopical study revealed that each cell was elliptical, budding present but no ascospore and no ballistospore. All these morphological and microscopical characteristics were at per with characteristics of *Candida famata* shown in the key of Barnetts et al. [1].

Table 1. Morphological and microscopical characteristics of yeast isolate

Characteristics	Yeast Isolate
Colour	White
Surface	Round
Margin	Undulated
Elevation	Convex
Cell- shape	Ellipsoid.
Ascospore	Absent
Ballistispore	Absent
Pseudomycelium	Absent
True mycelium	Absent

The biochemical characterization of the isolated yeast isolate shown on Table 2, indicated that this isolate gave positive result (growth) to D-glucose, D-galactose, L-sorbose, D xylose, sucrose, maltose, trehalose, cellobiose, lactose, rafinose, inulin, glecerol, D-mannitol of carbon assimilatory test while negative (no growth) to starch, myoinositol, 2 keto-D-glucose, D-glucoronate, succinate, citrate, methanol of carbon assimilatory test. It did fermented in maltose but in other carbon sources fermentation was weak or not happened. This isolate did not grow in nitrate or shown delayed or weak growth in nitrite and L- lysine. Regarding the effect of temperature, it could growth from 25 -35ºC while it did not growth at temperature from 37ºC and above. This isolate was sensitive to 0.01% of cyclohaxamide.

All these biochemical characteristics compared with the standard keys of yeasts [1,2,13,14] suggested that the yeast isolated from toddy palm belongs to *Candida famata* [Fig. 1].

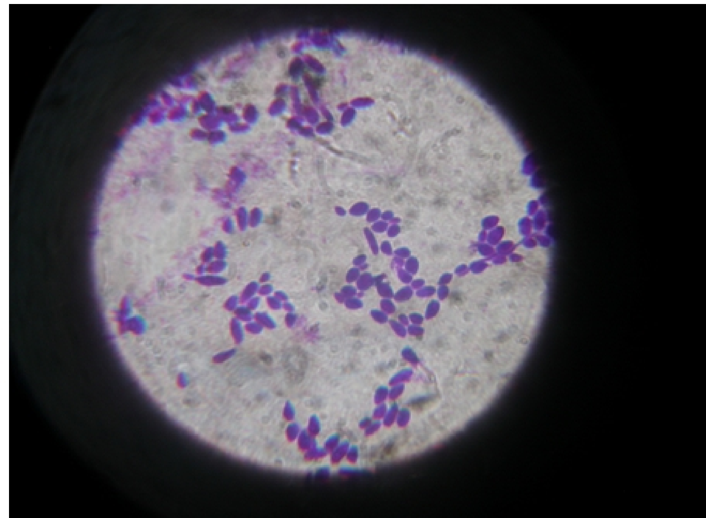

Fig. 1. Microscopic view of yeast isolate *(C. famata*) (10xX100x).

Table 2. Biochemical characteristics of yeast isolate using carbon assimilating tests and other tests

Sr. No.	Carbon assimilation test	Yeast Isolate	Sr. No	Carbon assimilation test	Yeast isolate
1.	D-glucose	+	26	Butane 2,3-diol	N
2.	D-galactose	+		Nitrogen assimilating tests	
3.	L-sorbose	+	27	Nitrate	-
4.	D-xylose	+	28.	Nitrite	D,W
5.	D-arabinose	D ,W	29.	L-Lysine	D,W
6.	L-ramnose	-	30.	Cadaverine	N
7.	Sucrose	+	31.	Glucosamine	N
8.	Maltose	+		Fermentation in carbon source	
9.	α,αTrehalose	+	32.	D-glucose	W
10.	Cellobiose	+	33.	D-galactose	-
11.	Lactose	+	34.	Sucrose	-
12.	Raffinose	+	35.	Maltose	+
13.	Inulin	+	36.	Lactose	-
14.	Starch	-	37.	Inulin	N
15.	Glycerol	+		Growth at different temperatures	
16.	D-glucitol	+	38.	25ºC	+
17.	D-mannitol	+	39.	30ºC	+
18.	Myo-inositol	-	40.	35ºC	+
19.	2-Keto-D-glucose	-	41.	37ºC	-
20.	D-Glucuronate	-	42.	40ºC	-
21.	Succinate	-		Different Tests	
22.	Citrate	-	43.	Diazonium blue -B	-
23.	Methanol	-	44.	Urea hydrolysis	-
24.	Ethanol	D	45.	Cyclohexamide (.01%	-
25.	Propane 1,2- diol	N	46.	Cyclohexamide (0.1%)	-
			47.	Starch formation	-

Responses: +=Positive growth; - = Negative (no growth) ;W=weak growth ;w/-weak or negative; W/+= weak or positive N=Not determined; D= Delayed growth ;VW=Very weak growth ;

Simillarly *C.famata* and other species of this yeast *(C. tropicalis, C.krusei* and *C.valida*) were isolated from toddy palm by other workers [7,16]. *Candida* sp are anamorphic yeasts. *Candida* sp was isolated and characterized from palm syrup, molasses, toddy and grapes in India [17] and moreover, *Candida famata* was isolated from the fruit surface of *Syzygium cumini* L [12]. *Candida famata* was reported as antagonistic to *Penicillium digitatum* and biocontrol agents of many post harvest diseases of fruits and vegetables [18,19,20]. Recently *Candida famata* was reported to be one emerging human pathogen and some yeasts are emerging opportunistic animal pathogen [21]. Desnos-Ollivier et al.[22] reported *C. famata* is a rare human pathogen. *C. famata,* while frequently isolated from air, soil, water, plant material and animals, and human and animal faeces, has been found to be a very rare aetiological agent in disease processes in animals and humans. A case of lifelong episodic diarrhoea in a dog that might have been aggravated by colonisation of the intestinal mucosa by *S. cerevisiae* and *C. famata* is reported [23]. In animals it has been isolated from the

udder of cows with mastitis, genital secretions of ruminants, the mouth of vitamin A-deficient pigs, and a fungal arthritis of the fetlock in a horse [24]. Therefore, *Candida famata*, are being recognized as potential biocontrol agent of post harvest diseases of fruits and vegetables and on the other hand it is being emerging pathogens that cause several types of infections in humans and animals. Under this situation very quick and perfect the identification of this fungus is urgent. In our experiments for identification of yeast, all morphological, microscopical and biochemical tests took one week. The total time required for identification of *Candida* species using species-specific PCR is less than 5h including 2h for DNA extraction, 1h and 40 min for PCR and 40min agarose gel electrophoresis. It is considered rapid as the identification can be done within a working day, as compared to conventional biochemical tests which require more than 5 days [25]. Detection and identification of fungal DNA by PCR is one of the most powerful and popular tools for the early detection and identification of pathogenic fungi, including *Candida* species [26]. In this sense, extraction and purification of DNA of *Candida famata* and protocol for its quick molecular or PCR identification are very much necessary.

L 1 of the Fig.2 showed the band obtained from manual method. This band was poor. L3 and L7 of the Fig.2 indicated the band obtained from DNA ladder and kit method respectively. The band of L7 (Kit method) was very prominent, bright and its size was more greater than 3000 kb as indicated by DNA ladder. The amount of gDNA extracted by kit method is 62ng /10 µg while it was 35ng/10µg by manual method. It was done by Quantifying soft wire (Biorad). Opara et al. [27] also obtained good result using kit method for DNA extraction.

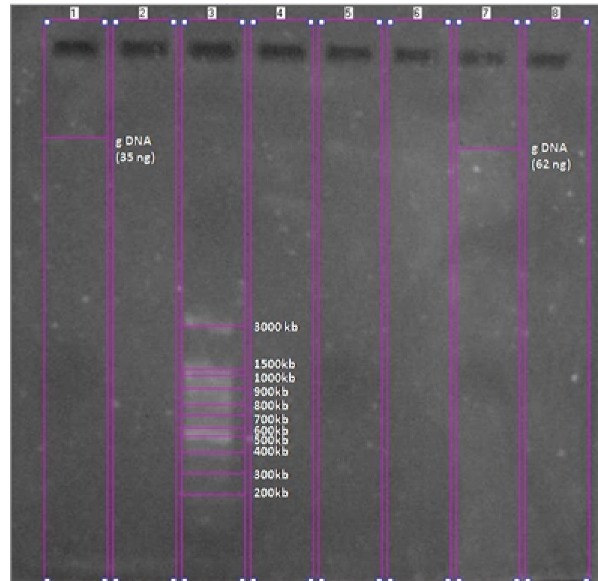

Fig. 2. DNA band observed from manual methodology (L1), from Kit method (L7) and DNA ladder (L3)

Cell lysis is the main step for efficient recovery of DNA. Nucleic acids must be solubilized from cells or other biological material. Conventional method for DNA extraction from yeast cells mainly include cell lysis and either enzymatic degradation or treating with glass beads. This method requires addition of precipitation solution (ethyl alcohol) and TE buffer in two

times which slows down the whole protocol and extraction of large number of DNA was not properly handled. So, this method is time consuming and it is inconvenient way to extract the high quality DNA. This was done according to the published keys [27].

On the contrary the kit (Uniflex^{TM} DNA isolation Kit) method belongs to basic DNA extraction protocol which include cell lysis, enzymatic treatment, phenol-chloroform and precipitation. The purity of DNA was checked by spectrophotometric method and the absorbance ratios **(A_{260}/A_{280})** obtained ranged from 1.8 to 2.0. The absorbance value obtained by kit method was 1.79 where as the value obtained by manual method was 1.5.

So this kit method is much more convenient way to get pure and high quality DNA than the manual method.

4. CONCLUSION

Toddy is one of the most important habitat of yeast, particularly *Candida famata* and this yeast is highly antagonistic to *Penicillium digitatum* early reported; on the other hand it is an emerging pathogen of animals. Isolated yeast from toddy was identified as *Candida famata.* The genomic DNA of *Candida famata* was extracted purely by Uniflex^{TM} DNA isolation Kit. This method was better and more convenient than manual method. This kit method for extraction of genomic DNA would be very useful for molecular or PCR identification of this yeast or other yeasts .

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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