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RESEARCH ARTICLE

FATTY ACID PROFILING OF ISOLATED TODDY YEASTS AND THEIR MOLECULAR IDENTIFICATION

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ABSTRACT

Toddy is a very well-known alcoholic beverage in south India and also knownin parts of Srilanka, Indonesia, Thailand, Africa, and South America. It is obtained through the natural fermentation of various palm saps like *Cocosnucifera*, *Borassus flabillifer*, *Phoenix sylvestris,* Sap is obtained by tapping the unopened spadix of the palm popularly known as neera. It is reported to be highly nutritive and a good digestive agent. However, it is highly susceptible to spontaneous fermentation, initially alcoholic, followed by acidic fermentation. The main products of fermentation are lactic acid, ethanol and acetic acid, and the fermented neera is known as toddy. The fermenting organisms are dominated by yeasts, particularly *Saccharomyces cerevisiae,* these organisms contribute to flavours, flavour is due to fatty acid, where in sap yeast utilise available sugars producing alcohol and fatty acids,which contribute flavour to toddy. In the present work toddy samples collected from the local area and the yeastswere isolated using chromogenic agar to eliminate to opportunistic *candida* species if present. The isolates were purified then identified through molecular and biochemical tests, the identified yeasts were *Pichia manshurica-H4S7K13, Saccharomyces cerevisiae Y5-3, Saccharomyces cerevisiae MUCL-51248*.They were analysed for fatty acid profiling using GC and MS analysis. The major fatty acids were 9-Octadecenoic acid methyl ester, 7-Octadecenoic acid methyl ester, Hexadecanoic acid methyl ester, Hexanedioic acid, bis-(2- ethylhexyl)ester', E -15 heptadecenal, 9-hexadecenoic acid methyl ester, 1-nonadecene, 1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester, these yeasts can be used in feed additives for aflatoxin binding and animal health improvement

INTRODUCTION

Toddy is a very well-known alcoholic beverage in south India enjoyed by people in parts of Srilanka, Indonesia,Thailand,Africa, and South America. It is consumed mainly by the low socioeconomic strata of society.It is obtained through the natural fermentation of various palm saps Coconut palm, *Cocos nucifera* L., Palmyrah palm (*Borassus flabillifer* L.),and wild date (*Phoenix sylvestris* L.). The sap is obtained by tapping the unopened spadix of the palm. Sap is traditionally tapped from the tree in an organized manner (Baliga and Ivy, 1961; Nathanael, 1966). It is reported to be highly nutritive and a good digestive agent (Devdas, Sundari, and Susheela, 1969; Lata and Kamala, 1966).The sap is highly susceptible to spontaneous fermentation, initially alcoholic, followed by acidic fermentation (Iwuoha and Eke, 1996; Odunfa, 1985). Main fermentable carbohydrate in palm sap is sucrose (12-15%) more than half of which is fermented by a mixed natural micro flora with in 24hr. to produce: lactic acid,ethanol and acetic acid (Shamala and Sreekantiah, 1988).

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The fermenting organisms are dominated by yeasts, particularly *Saccharomyces cerevisiae* (Okafor, 1974; Sanni, 1993).The typical flavour development of toddy is mainly from the sap and organism involved in fermentation (Borse *et al,* 2007). To be able to exploit the intrinsic characteristics of yeast involved in toddy fermentation, it is necessary to investigate the physiological attributes of yeast isolates. This work aims at screening and identifying the isolates by molecular methods and fatty acid profiling to understand the contribution of organism to the flavour of toddy. And use in feed industry as feed additive for aflatoxin detoxification.

MATERIALS AND METHODS

Toddy was collected from rural Mysore area from the local vendors. The samples were collected in sterile sample collecting pouches and stored at 4ºC till further analysis. Samples were screened for yeasts on Candida Chrome Agar media. Purple and white colour colonies isolated and purified. Whereas green colonies were rejected because those colonies are considered as *Candida* species. The selected isolates were then purified by serial streaking, and individual colonies were analysed for alcohol production and for fatty profiling.

Organism Identification

DNA was isolated from the culture based on the protocols provided by the company. Its quality was evaluated on 1.2% Agarose Gel. Fragment of D1/D2 region of LSU (Large subunit 28S rDNA) gene was amplified by PCR from the above isolated plasmid DNA. A single discrete PCR amplicon band of 650 bp was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The sequence obtained was aligned using online BLAST aligner software and identified using homology model.

Estimation of alcohol

A method based on that Caputi *et al.,* (1968), was performed to estimate the alcohol percentage. Sample (2 mL) was taken in 50mL distilled water in a distillation flask and was distilled at 65^{o}C. 15 mL of the distillate was collected in a conical flask and chromic acid (25 mL) was added. Volume made up to 50 mL with distilled water. The conical flasks were incubated at 60^{o}C on a water bath for 30 minutes. The solutions were brought to room temperature and the optical density was read at 600 nm spectrophotometrically. The alcohol percentages of the samples were calculated using the standard graph produced using absolute alcohol in the range of 0-15% and subjected to the same conditions of distillation and estimation.

Lipid analysis

Gravimetry The yeast cultures were grown on YEPD broth for48hours at ambient conditions and yeast biomass is harvested by centrifuging the cultures at 8000 rpm for 10 minutes. The harvested biomass was then freeze dried. The dried biomass was weighed. Lipid was extracted from 50 mg of dry biomass. The dry biomass was ground in a pestle and mortar with 0.5µglass beads using extraction solvent consisting Chloroform: methanol (2:1 ratio). Extraction was carried out thrice and the solvent layer was pooled. The extract was filtered using What man Filter paper No 1. The crude extract was passed through anhydrous Sodium Sulphate in order to remove the moisture .The extract was dried using a rotary evaporator and residues of solvent was removed using a jet of nitrogen gas. The lipid content obtained was expressed as % w/w with respect to the biomass quantity taken. (Christie, 1982). Lipid % = lipid content/biomass taken for extraction x 100

Fatty Acid Methyl Ester Preparation (FAME analysis)

The dry lipid extract was mixed with 2 ml of FAME reagent. The FAME reagent consisting methanol: acetyl chloride in 9.5:0.5 ratios was added to the sample and refluxed for 3 hours at 80°C in a water bath. The cooled sample was mixed with 5 ml Hexane in a separating funnel and the hexane fraction was collected the extraction was repeated twice and pooled. The pooled hexane layers was now washed with 5 ml of 2% Potassium Bicarbonate solution and extracted with hexane was repeated as mentioned in previous steps. The final hexane extracts was passed through anhydrous sodium sulphate to remove moisture and they are dried in rotary evaporator. The tube is finally flushed with nitrogen gas and stored in -20°C. Before injecting into GC/GC-MS the sample samples were dissolved in HPLC grade hexane and centrifuged at 10000 rpm for 5 minutes to remove undissolved particles.

GC parameters: Model: Shimadzu GC 2010 plus.Column: Rtx 1 Bonded; poly (dimethylsiloxane), 30m length, 0.32mm ID, 0.25 µm film thickness, Injector Port Temperature: 250 ºC; Column Temperature Program: 120°C (2 min hold) to 280^{o}C (10 min hold) with 5^{o}C/min rise Detector (FID) Port temperature: 280°C using nitrogen as carrier gasat 1ml/min. flow rate.(Dayananda *et al*., 2006) The standard Fatty acid mixtures and FAME standards were procured from SIGMA laboratories. The fatty acid profile was identified by comparing the retention time of each fatty acid with the standard. The fatty acid profile was further confirmed by GCMS analysis and compared with the NIST library data base

GC-MS Parameters: Model: PerkinElmer Instruments, USA Column: Rtx 1 Bonded; poly (dimethylsiloxane), 30m length, 0.32mm ID, 0.25 µm film thickness, Injector Port Temperature: 250 ºC Column Temperature Program: 120°C (2 min hold) to 280^{o}C (10 min hold) with 5^{o}C/min rise Detector (FID) Port temperature: 280°C Nitrogen gas at flow rate of 1ml/min warmed as carrier gas. Injector and Detector port temperatures were 220°C and 280°C respectively. The mass spectra were recorded under electron impact ionization at 70 eV electron energy with a mass range from 40-600 at a rate of one scan/s. The constituents were identified by comparing retention times of the GC peaks with those of reference compounds run under identical conditions and by comparison of retention indices with literature data (Adams, 2001; Davies, 1990; Jennings and Shibamoto, 1980), and fragmentation patterns in mass spectra were matched with those of the NIST62-LIB library and published mass spectra (Adams, 2001; Ten Noever de Bravw, Bovwman, Gramberg and La Vos, 1988).

RESULT AND DISCUSSION

The yeast diversity of toddy was enumerated using candida chrome agar. The non-candid isolates were subjected to biochemical test and alcohol production, isolates with distinct characters and alcohol production abilities were taken for further studies. The isolates were cultured on YEPD broth for 48 hours and the biomass was subjected to lipid analysis standard culture *Saccharomyces cerevisiae* 2375 was procured from Microbial Type Culture Collection centre was used as control for experiment maximum amount lipid was produced by *Saccharomyces cerevisiae* 2375(8%) and minimum by i.e *Saccharomyces cerevisiae, strain MUCL 51248*- (5.6%) *Pichia manshurica* (6.6 %), the other two isolates produced *Saccharomyces cerevisiae strain Y5-3*– (6.6%) lipids on dry weight basis.

Organism Identification

Molecular identification was carried out amplifying the concerned region of 28 r DNA was isolated from the respectivecultures. Its quality was evaluated on 1.2% Agarose

Gel, a single band of high-molecular weight DNA has been observed. Fragment of D1/D2 region of LSU (Large subunit 28S rDNA) gene was amplified by PCR from the above isolated plasmid DNA. A single discrete PCR amplicon band of 650 bp was observed when resolved on Agarose Gel .

Table 1. Lipid production by various isolates (% W/W dry weight)

Isolates	% of lipids	% Alcohol production
Pichia manshurica	6.6	ND
Saccharomyces cerevisiae, Strain MUCL 51248	5.6	13
Saccharomyces cerevisiae Strain Y5-3	6.6	7.5
*Saccharomyces cerevisiae*2375	8	4.5

The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Consensus sequence of 591bp of D1/D2 region of 28S rDNA gene was generated from forward and reverse sequence data using aligner software. The culture was identified as *Pichia manshurica, isolate H4S7K13* (GenBank Accession Number: FM180542.1).

Consensus sequence of 588bp of D1/D2 region of 28S rDNA gene was generated from forward and reverse sequence data using aligner software. The culture was identified *Saccharomyces cerevisiae, strain MUCL 51248* (GenBank Accession Number:FN393977.1). Consensus sequence of 607 bpof D2 region of 28S rDNA gene was generated from forward and reverse sequence data using aligner software.The culture was identified *Saccharomyces cerevisiae strain Y5-3* (GenBank Accession Number:HQ711330.1) On further characterization of lipids it was observed that *Saccharomyces cerevisiae, strain MUCL 51248* and *Saccharomyces cerevisiae* 2375 the major lipid produced palmitoleic (Table 1). However the other *saccharomyces cerevisiae*, had oleic acid as the major fatty acid component oleic acid was also the second major component in other two species of *Saccharomyces* used in the study, *Pichia manshurica* had palmitic has major lipid molecule closely followed by linoleic, the other major fractions were myristic, palmitoleic with oleic being the next major fatty acid only *Saccharomyces cerevisiae, strain MUCL 51248* had 5% of its fatty acid in the form of erucic acid and standard culture *Saccharomyces cerevisiae 2375* has linoleic and arachidic acids which are the normal finger prints of plant lipids. In the present analysis more than 97% of the fatty acids constituents were identified. Even though all three strains were fairly resistant to alcohol, the production capacity varies percentage of alcohol production given in Table 1, *Saccharomyces cerevisiae, strain MUCL 51248*(ITB) being the most promising and the least production was observed in *Saccharomyces cerevisiae strain Y5-3*(KTP), *Saccharomyces cerevisiae* (2375*), Pichia manshurica, isolate* H4S7K13 (ITW). Earlier (Borse *et al.,* 2007) had reported 2.56% of alcohol in

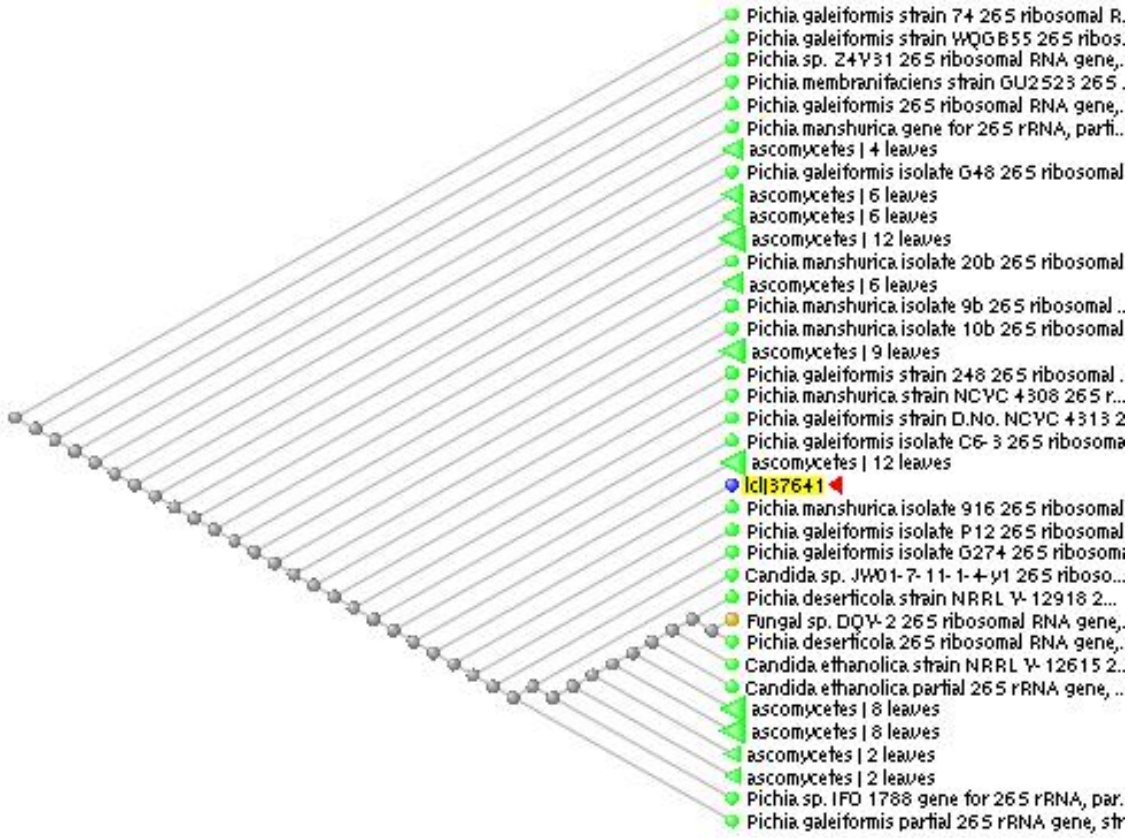

Figure 1.Phylogenic tree of *Pichiamanshurica, isolate H4S7K13*(ITW)

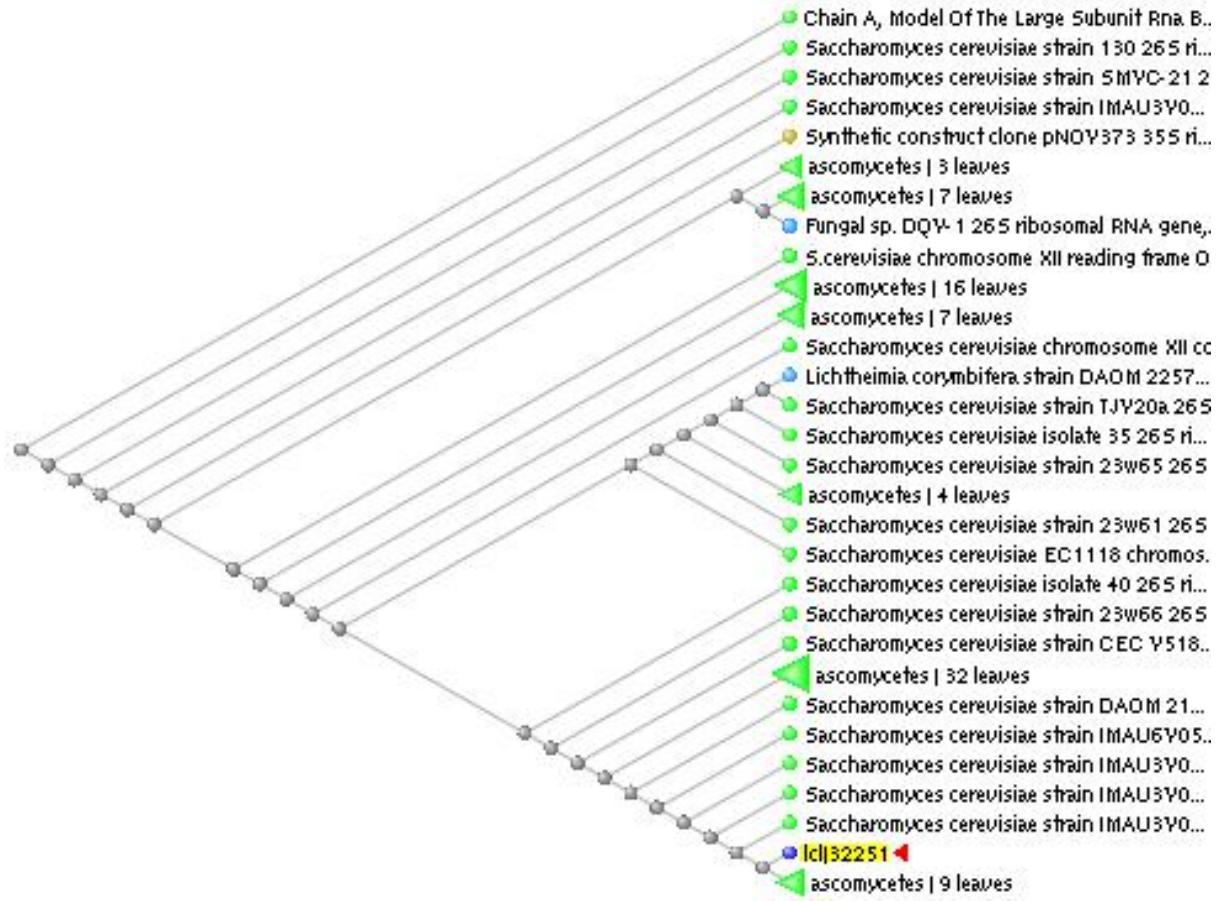

Figure 2. Phylogenic tree of *Saccharomyces cerevisiae, strain MUCL 51248*(ITB)

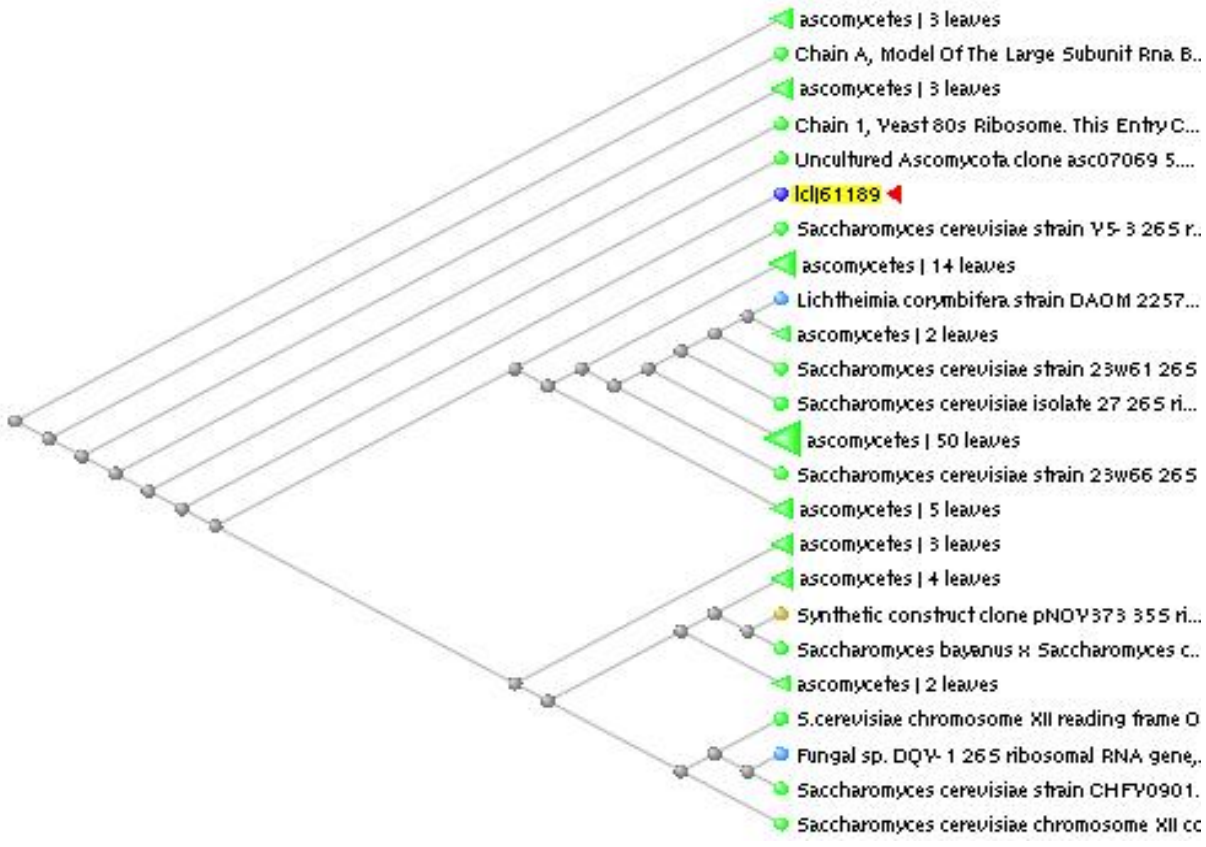

Figure 3. Phylogenic tree of *Saccharomyces cerevisiae strain Y5-3*(KTP)

Table 2. Major Lipds produced by isolates

S No.	Compound	Fatty acid	Quantity (%)			
			ITB	ITW	KTP	2375
1	C 12:0	Lauric acid	0.676	0.451	--	--
2	C14:0	Myristic acid	1.284	1.306	16.464	1.842
3	C16:1	Palmetoic acid	44.92	12.82	16.464	44.605
4	C16:0	Palmitic acid	8.044	18.26	23.439	13.130
5	C18:2	Linoleic acid	--	20.01	20.756	1.254
6	C18:3	Linolenis acid	--	7.176	1.270	--
7	C18:1	Oleic acid	36.1	33.436	15.080	29.202
8	C18:0	Stearic acid	3.633	6.539	0.113	4.966
9	C20:0	Arachidic acid	--	--	6.410	--
10	C22:1	Erucic acid	5.34			
11	C22:0	Behenate acid	--	--	--	4.997

toddy Fatty acids impart body to toddy. In the present experiment it has been noticed that *Saccharo myces* mainly contributed to the alcohol in toddy. As the alcohol percentage increases the alcohol resistant form tend to dominate. Hence it was considered that the fatty acids produced by the growing cultures contribute to the lipids in toddy. Sap of palm when fresh has very low level of lipids (Gupta, Jain and Shankar, 1980). Earlier working with Neera flavour (Borse *et al.,* 2007) had observed that the flavour profile complete change after fermentation. This is attributes to the fermenting organism mainly yeasts.

Conclusion

Alcohol production toddy is an in practice. Normally toddy has alcohol from 7-12%. In the present study the cultures produced alcohol within that range and *pichia* has not produced which was a potential candidate for our present work, which can be used as feed additive. The major fatty acids in all cultures were observed Oleic acid, followed by Palmitileic and palmitic acids. The astringency and harsh note of the toddy could be due to the increased amounts of acids, such as palmitoleic acid and dodecanoic acid, along with higher concentrations of ethyl alcohol and ethyl esters.

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