Characteristics of some traditional Vietnamese starch-based rice wine fermentation starters (men)

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Abstract

In the Mekong Delta region of South-Vietnam, wine from purple glutinous rice is particularly interesting because of its sherry-like taste and flavour and its attractive brown-red colour. It is manufactured at home or by small cottage industries, using traditional solid-state starters (*Men*). With the objective of improving the knowledge about the functionality of traditional *Men*, this study deals with the properties and composition of 29 samples of Vietnamese commercial rice wine starters. We selected 6 rice wine starters for their superior ability to liquefy cooked rice, high ethanol accumulation, and production of attractive flavour and colour in the resulting wine. Ethanol contents reached 12 g/100 ml, a sweet alcoholic fragrance was noticed and the wine colour varied from red to lightly brown. Total mould, yeast and bacteria counts in *Men* were 3.4–6.0, 5.8–7.2 and 2.6–6.2 log CFU/g of dry weight sample, respectively. A total of 119 microbial strains, comprising 53 moulds, 51 yeasts and 15 bacteria, was isolated. Mould isolates with excellent functionality were identified as *Amylomyces rouxii*, *Amylomyces* aff. *rouxii* (an atypical form of *A. rouxii*), *Rhizopus oligosporus* and *Rhizopus oryzae*. Yeast isolates with excellent fermentation properties were all identified as *Saccharomyces cerevisiae*; other, less functional isolates were identified as *Candida glabrata* and *Pichia anomala*.
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Keywords: Fermentation starter; *Men*; Moulds; Yeasts

1. Introduction

Like other traditional fermented alcoholic beverages in other East-Asian countries (Basuki et al., 1996; Hesseltine, 1983; Nout & Aidoo, 2002), rice wines are popular in Vietnam. The manufacture of rice wine can be characterized as the conversion of rice (*Oryza sativa* L.) by physical, microbiological and biochemical operations including steaming, inoculation with starter, mashing and fermentation. The alcohol content of Vietnamese rice wines varies and can reach up to 15 ml/100 ml (about 12 g/100 ml); by distillation, products with approximately 50 ml/100 ml (about 40 g/100 ml) alcohol can be obtained.

In Vietnam, the production of rice wine is a source of income for farmer families in rural areas. In Vietnamese,

Ruou means wine. In the north and south, we can find *Ruou De* or *Ruou Nep*: these are fermented from rice or glutinous rice, respectively, followed by distillation. In mountainous districts, such as Da Lat, Buon Me Thuot, and Dac Lac, an ethnic minority (*Thuong* people) produce *Ruou Can* that is fermented from rice, maize or cassava, with or without distillation.

In particular in the Mekong Delta of the south, *Ruou Nep Than* (purple glutinous rice wine) is made from purple glutinous rice without further distillation. It is commercialized in two qualities: crude cloudy wine containing sediment, and clear filtered wine. Its manufacture follows a traditional fermentation process in which powdered starch-based starter is mixed with steamed gelatinized purple glutinous rice, which is then incubated under ambient conditions. After an initial period of uncontrolled aerobic fungal solid-state fermentation, the now moulded mass is mixed with water, and is allowed to undergo

submerged alcoholic fermentation. After filtration, the final product is a clear purple liquid containing a mixture of residual glucose, accumulated ethanol and other soluble matter.

Mostly the alcohol content in the final product is approximately 7–10 ml/100 ml (about 6–8 g/100 ml), which would be insufficient to preserve the wine. Enrichment with (distilled) rice wine alcohol is often practiced to strengthen the liquor and enhance its shelf-life. The yield, flavour and overall acceptability obtained with these uncontrolled fermentation operations vary to such an extent that this is considered as a problem by local producers.

Local winemakers are aware that the choice of starter tablets, called *Men* in Vietnamese, influences the yield and quality of wine. Three major microbial groups namely moulds, yeasts and bacteria (Hesseltine, Rogers, & Winarno, 1988; Lim, 1991; Steinkraus, 1989) are encountered in traditional rice wine starters. It is generally held that moulds are the major producers of amylases that degrade starch into fermentable sugars, and that the yeasts convert the sugars to alcohol. Bacteria, mainly lactic acid bacteria (LAB), most likely occur as opportunistic contaminants. However, little is known about the comparative functionality of fungal species in these starters. Our research interest focuses on the role of individual moulds and yeasts during starch degradation and alcoholic fermentation, in a view to develop superior defined mixed starters at a later stage.

Three main kinds of Vietnamese traditional starters can be distinguished (Luong, 1998; Phuc, 1998): (a) without and (b) supplemented with oriental medicinal herbs and (c) starters supplemented with leaves containing aromatic essential oils. In the Mekong Delta region, starters of type (b) predominate. The types and quantities of herbs used may vary according to the different local producers; however, some commonly used oriental herbs in starter manufacture are: "Nhuc Dau Khau" (Mace: *Myristica fragrans* Houtt), "Cam Thao" (Licorice root: *Glycyrrhiza uralensis* Fish), "Te Tan" (Ginger: *Asarum sieboldii* Miq.), "Thao Qua" (Cardamom: *Amomum tsao-ko* Crev. et Lem.), "Tieu hoi" (Fennel: *Foeniculum vulgare* Miller), and "Dinh Huong" (Clove: *Syzygium aromaticum* L.). The latter supplements are used to produce fragrance and are believed to suppress the growth of undesired microorganisms in the final products.

Taking into consideration the situation of Vietnam in which each locality has its own way of producing starch-based rice wine starter, depending on available ingredients and preferences, the objective of this study is to pave the way for upgrading the traditional starter tablet technology for winemaking. This paper presents the functionality of a number of Vietnamese rice-wine starters in terms of their alcohol production, with rice saccharification and alcoholic fermentation stages as crucial assessment criteria, and identifies representative functional moulds and yeasts.

2. Materials and methods

2.1. Sample collection

Twenty-nine samples of dry commercial traditional rice wine starters were obtained from small-scale factories and markets in different provinces. Sampling was focused particularly on the leading producers in the Mekong Delta region. All starters were classified as type (b) (see Section 1). The starters were subjected to microbiological analysis immediately after collection. Samples were stored at 4 °C prior to screening for their functional properties.

2.2. Fermentation test

We adapted the traditional process for making rice wine for use at laboratory scale, as follows: (step #1) fifty grams of purple glutinous rice and 60 ml of distilled water in a 500-ml conical flask covered by a cotton plug were soaked (#2) for 4 h at room temperature (25 °C). After soaking they were steamed (#3) in an autoclave for 1 h at 100 °C. The gelatinized rice paste was cooled to 35–40 °C (#4), then inoculated and mixed well with 2 g of powdered starter to be tested (#5). After solid-state aerobic fungal fermentation during 3 days at 30 °C (#6), 70 ml of sterile water was added (#7) to the moulded mass to allow for submerged alcoholic fermentation for 7 days at 30 °C in the same flask, closed with a water lock (#8). All of the fermented rice mass was homogenized (#9) using a Stomacher Lab-blender (Seward 400, Emergo, England) and clear liquid was harvested after centrifugation (#10) at 3660 rpm for 20 min (Mistral 3000i; rotor windshield 43124-708 BS 4402, Leicester, United Kingdom); clear liquid (supernatant) was decanted (#11). Each sample was tested in triplicate.

2.3. Analytical methods

Numbers in brackets refer to the manufacturing stages mentioned in "Fermentation test" above. The pH after soaking (#2), after solid-state fermentation (#6) and after submerged fermentation (#8) was measured by digital pH metre (WTW pH 525). Flavour after (#6) and after (#8) was assessed subjectively by a small expert panel. Colour of clear final liquid was measured by Colorimeter Tricolor LFM3 (Dr. Lange), in which the L*, a*, b* system (DIN 6174, CIE-LAB standardized in 1976) was used. Glucose and ethanol after solid-state fermentation (#6) and after alcoholic fermentation (#8) were analysed by HPLC Organic Acid Analysis Column-Aminex HPX-87H Ion Exclusion Column 300 mm × 7.8 mm (BIO-RAD).

2.4. Microbiological analysis

For microbiological analysis, a sample of 1 g of each selected starter was transferred to a stomacher bag and homogenized with 99 ml sterile saline (NaCl 0.85 g/100 ml) in a Stomacher Lab-blender 400 for 1 min at high speed,

and appropriate serial dilutions were made in the same diluent. In total, 1-ml portions of the appropriately diluted suspension were mixed with molten (45 °C) medium and made into pour-plates.

Moulds were enumerated on Czapek-Dox Agar rendered selective for fungi by addition of oxytetracycline. The medium contained (g/l) sodium nitrate 2, potassium chloride 0.5, magnesium glycerophosphate 0.5, ferrous sulphate 0.01, potassium sulphate 0.35, sucrose 30, agar 20. To 900 ml of basal medium, 100 ml containing 100 mg sterile filtered solution of oxytetracycline was added. Oxytetracycline Glucose Yeast Extract Agar was used for yeast count. The ingredients (g/l) were yeast extract 5, glucose 20, agar 12. Oxytetracycline was added to this medium as above.

MRS medium was used to determine the LAB count. It contains (g/l) peptone from casein 10, meat extract 8, yeast extract 4, D(+) glucose 20, di-potassium hydrogen phosphate 2, Tween-80 1, di-ammonium hydrogen citrate 2, sodium acetate 5, magnesium sulfate 0.2, manganese sulfate 0.04, agar technical 12. To 1-l medium 2 g natamycin dissolved in 40 ml of sterile water was added to prevent fungal growth.

All plates were incubated at 30 °C for 2–4 days. The colonies that appeared after incubation were counted and calculated as colony forming units (CFU) per gram of dry weight sample.

2.5. Isolation and purification of microorganisms

In order to select for the most active strains in the various starters we isolated from fermentations initiated by these starters, instead of sampling the starters directly. For moulds: after completing the solid-state fermentation step (#6) of the fermentation test (mentioned above) a piece of moulded rice covered with mycelium was transferred and spread onto a plate containing sterile Czapek-Dox Agar supplemented with oxytetracycline, incubated at 30 °C for 3 days and resulting colonies were purified on Czapek-Dox agar.

For yeasts: after completing the alcoholic fermentation step (#8) appropriate dilutions were made into pouring plates with Oxytetracyclin Glucose Yeast Extract Agar which was incubated at 30 °C for 1 or 2 days. Colonies were purified on Glucose Yeast Extract agar.

For LAB: during step (#6) of the fermentation test, 1 ml of liquid released by the moulded mass was diluted and pour-plated into MRS supplemented with natamycin, incubated at 30 °C for 4 days, and colonies were isolated and purified on MRS agar.

2.6. Identification of moulds and yeasts

Mould isolates were identified based on morphological examination and cultural properties according to established taxonomic keys and descriptions (Hesseltine, 1991; Samson & Hoekstra, 2002).

Yeasts were identified on the basis of their cell morphology, biochemical and physiological growth properties (Rohm, Lechner, & Lehner, 1990; Middelhoven, 2000; Yarrow, 1998), as well as on internal transcribed spacer (ITS) sequences (Han, Kuijpers, Thanh, & Nout, 2004; Kurtzman, Boekhout, Robert, Fell, & Deak, 2003). Classification followed the taxonomic keys, references and BioloMICS software (http://www.cbs.knaw.nl) (Barnett, Payne, & Yarrow, 2000; Kurtzman & Fell, 1998; Smith, Yarrow, & Robert, 2002).

2.7. Statistical analysis

Experimental data were statistically analysed using Statgraphics Plus Version 5, Manugistics, Inc.

3. Results and discussion

3.1. Initial testing of starters

All tested starters converted purple glutinous rice into wine. After the first 3 days of fungal solid-state fermentation under aerobic conditions, liquefaction and saccharification were clearly noticeable: liquid of high glucose content was released by the moulded mass which showed abundant mycelial growth in certain cases. During the next stage of 7-day incubation under exclusion of air, the alcoholic fermentation took place as observed by gas formation.

Table 1 shows the mean values of three replications of liquefaction, pH, glucose, ethanol and colour measurements. The ethanol content obtained after alcoholic fermentation is an important criterion for functionality. Statistical analysis revealed significantly ($P < 0.05$) different alcohol levels between starters tested.

Differences of liquefaction patterns were also observed. There was a positive correlation between the extent of liquefaction and the production of ethanol. For starters 2, 6, 15, 20, 23 and 29 the results show that stronger liquefaction during the aerobic solid-state fermentation is associated with higher accumulated ethanol levels in the final product. This can be explained by strong activities of both moulds and yeasts in the microflora of the corresponding starters. However, high levels of reducing sugars were not necessarily completely converted into ethanol; for instance, in starters 9, 11, 14, 17, 18 and 27 high glucose, but low ethanol contents were obtained at the end of the alcoholic fermentation suggesting that the yeasts in these starters have limited abilities to accumulate ethanol.

The initial pH of soaked purple glutinous rice decreased from 5.6 to the range of 3.8–4.6 after the first 3 days of incubation and then remained quite stable around 3.5–4.3 until the end of the fermentation.

On visual inspection, the colour of the final products was described as red to lightly brown. The colour was also measured by Colorimeter using the L*a*b*-system. The

Table 1
Performance of Vietnamese traditional rice wine starters

Starter	Liquefaction of steamed rice[a]	pH		Glucose (g/100 ml)		Ethanol (g/100 ml)		Colour measurement[b] after wine filtration		
	After mould fermentation	After mould fermentation	After alcoholic fermentation	After mould fermentation	After alcoholic fermentation	After mould fermentation	After alcoholic fermentation	L	a	b
1	+ +	3.8	3.6	19.3	0.6	5.3	10.2 cdef[c]	50.7	27.5	17.1
2	**+ + +**	**4.1**	**3.9 b**	**12.9 b**	**0.1**	**8.2 c**	**11.0 b**	**43.8**	**30.6**	**20.4**
3	+	4.6	3.6	20.2	0.3	5.0	9.6 hik	45.8	29.7	20.8
4	+	4.4	3.6	17.9	0.5	4.8	9.7 ghik	45.6	29.8	22.0
5	+	4.6	3.5	18.6	0.4	4.9	9.8 fghi	42.8	31.8	22.4
6	**+ + +**	**4.6**	**4.2 a**	**21.7 a**	**0.1**	**5.6 e**	**11.2 b**	**42.5**	**33.1**	**22.2**
7	+	4.4	3.7	17.5	1.2	4.8	10.5 c	51.5	21.2	21.7
8	+ +	3.9	3.7	14.4	1.1	6.6	9.9 efghi	49.1	26.4	20.7
9	+	4.4	3.5	24.5	0.9	4.2	9.5 ik	48.4	29.6	19.1
10	+	4.4	3.5	24.1	0.7	4.5	10.0 defg	46.4	30.6	19.7
11	+ +	4.0	3.5	24.2	0.6	4.6	9.6 hik	49.4	26.8	18.8
12	+ +	4.1	3.9	4.4	0.7	9.6	9.8 fghi	54.5	20.8	17.7
13	+ +	4.1	3.9	22.3	0.6	5.4	10.3 cd	52.1	24.2	17.3
14	+ +	4.6	3.7	24.3	1.1	5.3	9.9 efgh	46.0	29.6	21.2
15	**+ + +**	**4.5**	**4.3 a**	**21.8 a**	**0**	**6.6 d**	**11.1 b**	**45.0**	**29.7**	**21.3**
16	+ +	4.5	4.1	18.4	0.2	7.6	10.3 cd	47.7	26.1	21.6
17	+	4.1	3.4	20.1	1.1	4.5	9.6 hik	47.2	30.4	18.9
18	+	4.2	3.7	24.3	2.3	4.9	8.8 l	49.1	27.1	20.5
19	+ +	4.6	4.1	18.1	0.4	5.8	10.3 cd	48.7	24.9	22.9
20	**+ + +**	**4.4**	**4.3 a**	**12.9 b**	**0**	**9.3 b**	**11.7 a**	**46.4**	**29.8**	**22.2**
21	+ + +	4.6	4.2	16.2	0.4	6.9	10.1 cdefg	50.8	22.2	22.1
22	+ + +	4.6	4.1	12.9	0.6	7.2	10.2 cde	48.6	26.2	20.9
23	**+ + +**	**4.4**	**4.3 a**	**9.5 d**	**0.1**	**10.1 a**	**12.0 a**	**44.6**	**29.5**	**19.7**
24	+	4.6	4.1	22.4	0.4	5.8	10.5 c	49.1	25.1	22.1
25	+ +	4.6	4.4	20.9	0.5	6.0	10.3 cd	54.9	18.8	21.0
26	+ +	4.5	4.3	13.5	0.6	7.5	10.2 cdef	54.8	20.0	19.9
27	+	4.6	3.6	20.6	1.1	5.8	9.4 k	46.8	27.7	21.6
28	+ +	4.6	3.6	20.2	0.9	5.4	9.5 hik	43.1	31.6	23.2
29	**+ + +**	**4.4**	**4.3 a**	**11.7 c**	**0.1**	**10.0 a**	**12.0 a**	**45.4**	**28.7**	**19.4**

[a]Levels of liquefaction ranging from + (little) to + + + (very much); starters bold are those selected for further study.

[b]Colour of clear liquid was measured by Colorimeter Tricolor LFM3, in which the L*, a*, b* system (DIN 6174, CIE-LAB standardized in 1976) was used (L = luminosity, a = red component, b = blue component).

[c]Means with different subscripts are statistically significant at the 95% confidence level.

results show that the choice of starters did not strongly influence the perceived and measured colour of the wine.

The flavour of the fermenting rice was recorded after 3 days and at the end of the fermentation. After 3 days, a sweet fragrance was dominant in all treatments except with starters 9 and 28 which caused a slightly sour flavour, whereas slight alcoholic flavour was noticed with starters 2, 12, 20, 23 and 29. The final products had a strong alcohol flavour in all cases, and in starters 1, 2, 3, 20 and 29, sweet odours still remained detectable.

During the fermentation process, glucose contents were high after the first 3 days, but were exhausted in most cases at the end of the fermentation. Concomitantly, ethanol levels increased, confirming expectations that starch will first be converted into sugars and these subsequently into alcohol. With starters 2, 12, 20, 23 and 29, glucose contents were rather low after mould fermentation but nevertheless they were accompanied by significant levels of ethanol. We

assume that in these cases, the yeasts had already started to convert glucose as soon as it was produced and continued exhausting it towards the end of the fermentation. In contrast, the yeasts in starters 9, 10, 11, 14 and 18 only weakly fermented glucose that had been formed at high levels, resulting in low ethanol levels and significant residual sugar in the final product. In the case of starters 2, 6, 15, 20, 23 and 29 we observed a complete fermentation, i.e. exhaustion of glucose. In all treatments, ethanol was detectable already after the first few days of the process, indicating that the production of rice wine using a starch-based starter is indeed the result of mixed and simultaneous fermentations. We also observed that all starters contained yeasts that were able to grow under aerobic conditions; however, the significant increase of ethanol levels later during the process shows that fermentation by the yeasts is favoured by anaerobic conditions.

These data show a considerable variation of starter functionality. Some starters (2, 6, 15, 20, 23 and 29) were considered of interest for further investigation because of their capacity for rice liquefaction, their significantly higher ethanol accumulation, as well as the flavour and the colour of their wines.

3.2. Microflora composition

Predominant groups of microorganisms were enumerated in the six selected starters and reported as log CFU/g of dry weight sample as shown in Table 2. Although it is known that mould counts represent their extent of sporulation rather than their viable biomass, in this case the use of CFU counts will be appropriate as the spore germination activity is essential for starter performance. Yeasts were present in the highest numbers (up to 7.2 log CFU/g starter). In general, LAB were present at relatively low levels compared to yeasts and moulds. In relation with

the performance of selected starters (Table 1), low pH can have resulted from the presence of higher numbers of LAB such as in starter 2; on the other hand, yeasts are also able to reduce the pH by their efflux of protons. In starters 2, 20, 23 and 29, glucose contents after mould fermentation were significantly lower in comparison with results in starters 6 and 15, and vice versa ethanol contents in the former starters were significantly higher. This may have been caused by a rapid conversion of glucose by the yeasts as soon as it was formed from starch. This would also partly explain the high yeast counts in starters. However, starter 6 is an exception, indicating that not only cell numbers but rather their species determine the functionality of the starters.

3.3. Identification of selected moulds and yeasts

A total of 119 strains, comprising 53 moulds, 51 yeasts and 15 bacteria, was isolated. This investigation focussed on improving performance, so the isolated fungi were subjected to a screening program of which the main outcomes are summarized in Table 3. Three main groups of moulds and yeasts were distinguished according to their starch degradation and ethanol production, respectively. Eight mould isolates expressing strong starch degrading ability that was evaluated by measuring diameters of clearing zones in rice-starch agar, were selected for further studies and identified as four species, namely *Amylomyces rouxii*, *Amylomyces* aff. *rouxii* (an atypical form of *A. rouxii*), *Rhizopus oligosporus* and *Rhizopus oryzae*. These species were also found earlier in amylolytic fermentation starters used in the production of alcoholic beverages in various Asian countries (Hesseltine et al., 1988; Nout & Aidoo, 2002). All species belong to genera of the Zygomycetes, which are commonly found in foods; these are confined to the order Mucorales. The moulds in this

Table 2
Total mould, yeast and lactic acid bacteria (LAB) counts (log CFU/g dry weight) of selected fermentation starters

Starter	Moulds			Yeasts			LAB		
	a[a]	b	Mean values	a	b	Mean values	a	b	Mean values
2	5.8	6.2	6.0 a [b]	7.4	6.8	7.1 a	5.8	6.6	6.2 a
6	5.0	3.6	4.3 d	7.0	6.6	6.8 bc	4.2	5.2	4.7 b
15	3.7	3.1	3.4 e	6.1	5.5	5.8 d	2.9	2.3	2.6 f
20	5.5	4.7	5.1 b	7.1	7.3	7.2 a	4.8	4.0	4.4 c
23	5.1	4.1	4.6 c	6.5	7.3	6.9 b	4.1	3.9	4.0 d
29	4.8	4.2	4.5 c	7.0	6.4	6.7 c	3.6	3.0	3.3 e

[a]a and b are duplicates.
[b]Means with different subscripts are statistically significant at the 95% confidence level.

Table 3
Summary of main outcomes of mould and yeast screening for their functionality and the identification of representative isolates

Group of isolates	Starch degradation by moulds[a]		Fermentative capacity by yeasts[b]		Identification of representative isolates	
	Number of isolates	Diameter of clearing zone (cm)	Number of isolates	Alcohol content (g/100 ml)	Moulds	Yeasts
Group 1	8	7.1–9.0	45	8.2–8.8	*Amylomyces rouxii* (3 strains); *Amylomyces* aff. *rouxii* (1 strain); *Rhizopus oligosporus* (1 strain); *Rhizopus oryzae* (3 strains)	*Saccharomyces cerevisiae* (5 strains)
Group 2	21	5.0–6.3	5	5.2–5.9	Not identified	*Candida glabrata* (1 strain)
Group 3	24	2.1–4.7	1	4.3	Not identified	*Pichia anomala* (1 strain)

[a]Moulds were inoculated on glutinous rice starch plates, incubated at 30 °C for 48 h, and the plates were subsequently flooded with 0.25% iodine solution.
[b]Fermentation tests were carried out on saccharified purple glutinous rice liquid, standardized at 20°Brix, in conical flasks with water locks. Analysis was done after 5 days of incubation at 30 °C.

order can produce alcohol under low oxygen conditions (Cronk, Steinkraus, Hackler, & Mattick, 1977). Similarly, the five randomly selected yeast isolates from group 1 that showed superior fermentation ability were identified and they all belonged to the species *Saccharomyces cerevisiae*. Although other yeasts such as *Saccharomycopsis fibuligera* (Knox, Preez, & Kilian, 2004) have been identified in rice starters as well, it is not surprising to encounter *S. cerevisiae*, as numerous studies document that the species *S. cerevisiae* is predominant in alcoholic fermented beverages (Barnett et al., 2000; Battcock & Ali, 1993). Besides, two representative isolates belonging to the other two groups that had the lower ethanol producing capacity were also included in the classification. They were identified as *Candida glabrata* and *Pichia anomala*. In fact, all these isolates originated from the six rice wine starters that had been selected for their superior ability to liquefy cooked rice, high ethanol accumulation, and attractive flavour and colour of the resulting wine. However, they performed very differently in starch degradation and ethanol production as well as belonged to different species. This is a consequence of the fact that original rice wine starters are not defined pure culture starters, but contain a mixed microflora. In further studies, the behaviour of high-performing strains as single or mixed pure cultures should be tested. The compatibility of moulds and yeasts is an important aspect for the production of starters with good quality. Therefore further research into the interaction of moulds and yeasts predominating in these starters will be required.

Acknowledgements

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