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ATF1 Gene Diversity Studies of *Saccharomyces Cerevisiae* Strains of North Western Himalayas

Keshani[1] and Mohit Garg[2]

[1]*Extension Scientist, Department of Microbiology, College of Basic Sciences and Humanities*
Punjab Agricultural University, Ludhiana, India
[2]*M.Sc. Student, Department of Microbiology, College of Basic Sciences and Humanities*
Punjab Agricultural University, Ludhiana, India

Abstract—*North Western Himalayan region is a rich repository of microbial diversity. Yeast strains were isolated from traditional alcoholic beverages of Lahual and Spiti, Bharmour and Bada Bangal region of North-Western Himalayas. Molecular identification by ITS region sequencing showed that all the yeast strains were Saccharomyces cerevisiae. After identification of yeast strains ATF1 gene mining was done. Multiple sequence alignment of ATF1 gene of indigenous brewing yeast strains with Saccharomyces genomic database (SGD) revealed nucleotide substitutions and difference of about 14 amino acids. MK680909 strain showed highest dissimilarity with other strains used in the study showing diversity in ATF1 gene of yeasts of North Western Himalayas.*

Introduction

During fermentation processes, yeast cells produce a broad range of aroma-active substances which greatly affect the complex flavor of fermented alcoholic beverages. While these secondary metabolites are often formed only in trace amounts, their concentrations determine the distinct aroma of these beverages. Flavor-active substances produced by fermenting yeast cells can be divided into five main groups: sulfur-containing molecules, organic acids, higher alcohols, carbonyl compounds, and volatile esters [7,9]. Of these, volatile esters represent the largest and most important group as they are responsible for the highly desired fruity character of beer and, to a lesser extent, other alcoholic beverages, such as wine.

The best-known enzymes involved in ester synthesis are alcohol acetyltransferases (AATases; EC 2.3.1.84). These enzymes catalyze the formation of acetate esters from the two substrates: an alcohol and acetyl-CoA. Purification of the acetate ester-synthesizing enzymes has led to the identification of three distinct AATases: AATase I, its closely related homologue Lg-AATase I, and AATase II. These AATases are encoded by *ATF1*, the *ATF1* homologue Lg-*ATF1*, and *ATF2* genes, respectively [3, 12-13]. While *ATF1* and *ATF2* are present in both *Saccharomyces cerevisiae* (ale) and *Saccharomyces bayanus* (lager) strains, Lg-*ATF1* is found only in *S. bayanus* strains [13]. Homology-based searches of the *S. cerevisiae* genome have not revealed other genes with homology to *ATF1* and/or *ATF2*. [11] have demonstrated that overexpression of *ATF1* in a commercial brewer's strain leads to significantly increased concentrations of isoamyl acetate and ethyl acetate in the beers produced. These results indicate that the expression level of *ATF1* is an important limiting factor for ester synthesis under industrial conditions. The variation in *ATF1* gene could also be revealed by organoleptic studies of these isolates and then comparing their profiles with variations observed at genetic level to obtain relevant information regarding the diversity in the indigenous isolates. Broad analysis on aroma and flavour production by different *Saccharomyces* species (*S. cerevisiae, S. kudriavzevii, and S. uvarum*) and their hybrids revealed significantly different expression levels of *ATF* genes for the production of higher alcohols and esters, which are key components of aroma and flavor in fermented foodstuffs [4,8]. Hence, this gene was targeted for studying functional diversity from an aromatic and flavor point of view at molecular level.

Material and Methods

Yeast isolation and maintenance of cultures

Samples were taken from Lahual & Spiti and Bharmour region of North Western Himalayas. Samples were collected under aseptic conditions. Serially diluted samples were plated on YEPDA plates. Plates were observed daily until colonies were appeared. Yeast colonies were purified by repeated streaking. Yeast isolates were preserved in 50% glycerol.

ITS region sequencing

ITS sequencing is the most extensively used technology for molecular identification. Sequencing of this ITS region was done with the help of commercial sequencing facility (Xcelris Labs Ltd., Ahmedabad, India). Editing and analysis of the ITS region of yeast isolates was done by using NCBI BLASTN program (http://www.ncbi.nih.gov/ blast) [1].

ATF1 gene mining

After identification yeast strains were selected for *ATF*1 gene mining. From the literature it was found that the gene consisted of 1578 bp long protein coding region of *ATF1* gene and after including the preceding promoter and TATA box it

was found to be 1988 bp. For amplification and sequencing, this 1988 bp region was In silcio divided into two overlapping sequences. The sequences were custom sequenced (ABI 3730xl automated sequencer) with both forward and reverse primers by a commercial sequencing facility (Xcelris Labs Ltd., Ahmedabad, India). The complete gene sequences were then recovered by aligning the overlapping regions of the obtained contigs. The homology search for these sequences was carried out using an on-line NCBI BLASTN program http://www.ncbi.nih.gov/blast [1]. By using multiple sequence alignment these gene sequences were compared with indigenous and exotic gene sequences of *Saccharomyces cerevisiae*. The amino acid sequences were deduced by using expasy tool. These were compared with multiple sequence alignment by MEGA X software program. All the phylogenetic analyses were conducted in MEGA X software program.

Results

Yeast isolation

Seven morphologically different yeast isolates were obtained from alcoholic beverages (*Gudanji* and *Sura*) collected from Lahual and Spiti, and Bharmour region of North Western Himalayas. Microscopic examination of the yeast colonies showed spherical coccus shape. Different sugars viz. dextrose, fructose, xylose, lactose, maltose and mannitol were used for sugar fermentation. Majority of yeast strains produces acid and gas for different sugars.

ITS region sequencing

ITS region sequencing was used for identification of yeast isolates. The sequencing was carried out using commercial facility i.e. Xcelris Labs Ltd., Ahmedabad. Universal primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) were used for amplification of ITS (ITS1-5.8S-ITS2) region and observed a product size of approximately 800 bp. Yeast isolates showed more than 98% similarity with *Saccharomyces cerevisiae* after analysis of the retrieved sequences using the NCBI, BLASTn program (Figure 1).

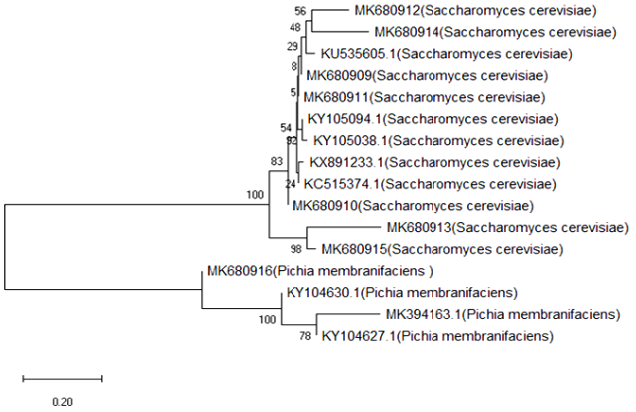

Figure 1: Phylogenetic tree showing clustering behavior of different yeast isolates on basis of their ITS region sequencing

ATF1 gene mining

In our study it was observed that the gene consists of 1578bp in open reading frame that encodes 525 amino acids. Protein coding regions of *ATF1* gene displayed a broad variation in *S. cerevisiae* strains. At different locations, 29 nucleotides substitutions were observed by multiple sequence alignments without any deletions or insertions within the gene of indigenous strains as well as in the *ATF1* gene sequence deduced from *Saccharomyces* genomic database (SGD).

In promoter region of the yeast strains no difference was observed. Amino acid sequences were deduced from the coding region of *ATF1* gene with the help of expasy tool. All the yeast strains showed variation in amino acids. Analysis of amino acid sequences of the *ATF1* genes exposed difference of about 9 amino acids (Table 1) among the indigenous yeast strains, proposing a great variation in aroma and flavor of fermented products. From these observations it was also found that most of the nucleotide substitutions leads to degeneracy of codons. MK680909 strain showed 4 amino acid variations

Table 1: Variation in amino acids among the different strains on basis of their nucleotide substitution

Yeast	Original	Replaced	Nucleotide Site no.	Original aa	Replaced aa	Aa Site no.
MK680914	CGG	GGG	79	R	G	27
MK680909	GTT	GGT	95	V	G	32
MK680909	TAT	GAT	106	Y	D	36
MK680909	TGT	TGG	174	C	W	58
MK680909	CAA	CCA	338	Q	P	113
MK680910	GAA	GCA	341	E	A	114
MK680914	CTG	ATG	349	L	M	117
MK680914/ MK680913	AAT	AAA	1173	N	K	391
MK680911	TCC	TTC	1547	S	F	516

Discussion

[10] isolated various microorganisms viz. Lactobacillus plantaru, Lb. casei, Enterococcus faecium, Pediococcus pentosaceus, Saccharomyces cerevisiae, Saccharomyces fibuligera, Pichia kudriavzevii, and Candida tropicalis from rice beer (chhang/lugri) made in the tribal belt of Lahaul and Spiti district of Himachal Pradesh. In our study we isolated all the strains of Saccharomyces cerevisiae from traditional alcoholic beverages of North Western Himalayas. [6] used internal transcribed spacer (ITS) region to differentiate S. cerevisiae strains isolated from fermented foods of North-Western Himalayas. ITS sequencing revealed that 18 yeast strains showed a little region based clustering. The strains used in this study were also identified using ITS region sequencing.

[5] reported that *ATF1* gene consisted 1566 bp in ORF that encodes 522 amino acids in native *S. cerevisiae* strains. However, in our study the ORF was found out to be of 1578 bp coding 525 amino acids as reported by [2]. *ATF1* gene plays an important role for the synthesis of esters in *S. cerevisiae* in both ale (top fermenting) and lager (bottom fermenting) yeast strains [11]. Yeast cells produces broad range aroma-active secondary metabolites in trace amounts that greatly affect the aroma and flavor of fermented alcoholic beverages. MK680909 strain showed highest dissimilarity with other strains used in the study showing diversity in *ATF*1 gene of yeasts of North Western Himalayas that further indicates diversity in esters.

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