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Comparative Study of the Effect of Temperature and pH on the Growth of Bio-flavor Producing Microorganisms *Kluyveromyces marxianus* and *Ceratocystis fimbriata*

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Abstract

Kluyveromyces marxianus and *Ceratocystis fimbriata* are two potential microorganisms that have been reported to produce fruity and floral aroma compounds during fermentation. In the present investigation, an *in vitro* comparative study was conducted using a liquid medium to investigate the effect of temperature from 20±2°C to 45±2°C and pH from 3 to 8 on the growth of both microorganisms. The results showed that the optimum growth of *K. marxianus* was found at 30°C while *C. fimbriata* showed the highest growth at 20°C. Similarly, the optimum growth of *K. marxianus* was found at pH 4.5 while *C. fimbriata* showed the highest growth at pH 6.5. The comparative analysis suggested that *K. marxianus* has a shorter lag phase and shows better growth at ambient temperature and pH compared to *C. fimbriata*. Thus, it is concluded that *K. marxianus* can be a better choice for the industrial production of bio-flavors.

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Introduction

Flavors play a vital role in the food and pharmaceutical industries. Due to increasing side effects caused by long-term usage of synthetic flavors, consumers are shifting choices towards natural or bio-flavors. Many recent studies reported that microorganisms are an excellent and economical source of producing bio-flavors [1]. Researchers have reported two microorganisms, *Kluyveromyces marxianus* (later referred to as *K. marxianus*), and *Ceratocystis frimbiata* (later referred to as *C. frimbiata*), to produce fruity and floral bio-flavor over three decades [2, 3]. *K. marxianus* is a budding yeast that grows up to 3-5 µm into spherical to oval shape [4]. *Kluyveromyces* is a 17-species genus producing fruity, floral terpenes, and 2-phenylethanol via de novo synthesis [1]. *K. marxianus* is a thermotolerant species with 70 min of generation time, shows growth at 52˚C and has a wide pH range acceptance with growth media having higher sugar content due to their capability to assimilate [5]. *C. frimbiata* is a fungal mold and is classified under Ascomycota with 42 reported species [6-9]. These fungi can survive on a decaying plant for over two years and in soil for over two weeks. *Ceracytosis frimbiata* produces floral and fruit-odor terpenes and is also a potential microbe for ester formation [1]. They are mainly grown on potato dextrose agar/slants and oatmeal agar and incubated in UV light at 21ºC for 15 days [10, 11].

These two microorganisms seem to be the most promising and potential fungi, which can grow in various solid substrates and produce several flavors and aroma compounds after fermentation. But so far, there is no comparative study done that reveals the best choice of microbial culture for industrial bio-flavor production among them. This study was planned to exploit the microbial culture for *in-vitro* assessment of the growth physiology of *K. marxianus* and *C. fimbriata* under different temperatures and pH conditions. This will help to understand, which microbial culture can withstand the harsh condition of industrial processing during fermentation and can synthesize the bio-flavor product.

Materials and Methods

Procurement of microbial cultures

Kluyveromyces marxianus (NCDC-39) was procured from the National Collection of Dairy Cultures (NCDC) of the National Diary Research Institute (NDRI), Karnal, India. The fungus strain, *Ceratocystis fimbriata* (MTCC-2281) was obtained from the Microbial Culture Collection Center of the Institute of Microbial Technology (IMTECH), Chandigarh, India.

Inoculum preparation

Kluyveromyces marxianus and *Ceratocystis frimbiata* were inoculated in separate sterile potato dextrose broth medium (PDB). A conical flask containing *K. marxianus* was incubated in a shaker incubator at 30±2˚C overnight [12]. While the liquid culture for *C. frimbiata,* was incubated at 25±2˚C overnight in a separate shaker incubator. Similarly, the subculture slants were stored in the refrigerator at 4°C, for further study.

Physiological studies

Comparative physiological studies such as the effect of different level of temperature and pH values on the growth of *K. marxianus* and *C. frimbiata* was carried out in PDB culture medium. Two sets of experiments were carried out for two different microorganisms, one set for *K. marxianus* and another set for *C. frimbiata.* Each set contains six conical flasks that consist of 20 ml of sterile media in 50 ml conical flask. All the conical flasks were inoculated with 1 ml of fresh microbial culture at the same time. After inoculation, each flask was cotton plugged and incubated in a shaker incubator for a specific time before observation. Microbial growth was analyzed by using a UV-visible spectrophotometer.

Effect of temperature on microbial growth

The influence of temperature on the growth of *K. marxianus* and *C. fimbriata* was studied at six temperature levels, *viz*., 20˚C-45˚C. About 20 ml of PDB was taken into six 50 ml conical flasks. Then each flask was sterilized at 121˚C for 20 minutes. After autoclaving, each conical flask was allowed to cool at room temperature, inoculated with 72 hrs fresh microbial cultures (1 ml) and then incubated at different temperature values starting from 20±2°C-45±2˚C in separate incubators. The microbial growth in the PDB was observed by its turbidity. The turbidity of the culture media was measured by a UV-visible spectrophotometer at the absorbance of 600 nm wavelength for *K. marxianus* culture (it is fungal yeast thus cell/ml was considered) and 405 nm wavelength for *C.*

fimbriata culture (it is a fungal mold thus spore/ml was considered) [13]. The data were analyzed statistically for significance using analysis of variance [14, 11].

Effect of pH on microbial growth

The influence of pH on the growth of *K. marxianus* and *C. fimbriata* was studied at six pH levels from 3-8. The pH of the PDB medium in each conical flask was regulated at respective levels with 1N HCl or NaOH by using a digital pH meter and then sterilized at 121˚C for 20 minutes. After autoclaving, each conical flask was allowed to cool at room temperature then each flask was inoculated with 72 hrs fresh microbial cultures and then incubated at 27±2˚C. The microbial growth in the broth culture media (turbidity) was measured by spectrophotometer and the absorbance as OD_{600} was recorded at an interval of 0, 5, 10, 15, 20, 25, 30, and 35 hours. Each experiment was carried out in triplicates. The average value of triplicate in a treatment was used as a measure for comparing *K. marxianus* growth and *C. fimbriata* growth separately [11].

The analysis of variance (two-way) was performed at a 5% level of significance using MS-excel. To study the significant difference between the growth of both the microorganisms in various temperature and pH levels, with respect to time. In addition, between temperature levels and time.

Results

Microbial growth at different temperatures

The impact of incubation temperature on the growth of *K. marxianus* and *C. fimbriata* is shown in Fig. 1. The average optimum growth of the yeast *K. marxianus* was noted at an incubation temperature of 30˚C with OD_{600} of 0.183. It showed that microbial culture enters an exponential growth after 4 hrs of incubation and the graph also shows that it reaches a stationary phase after 12 hrs of

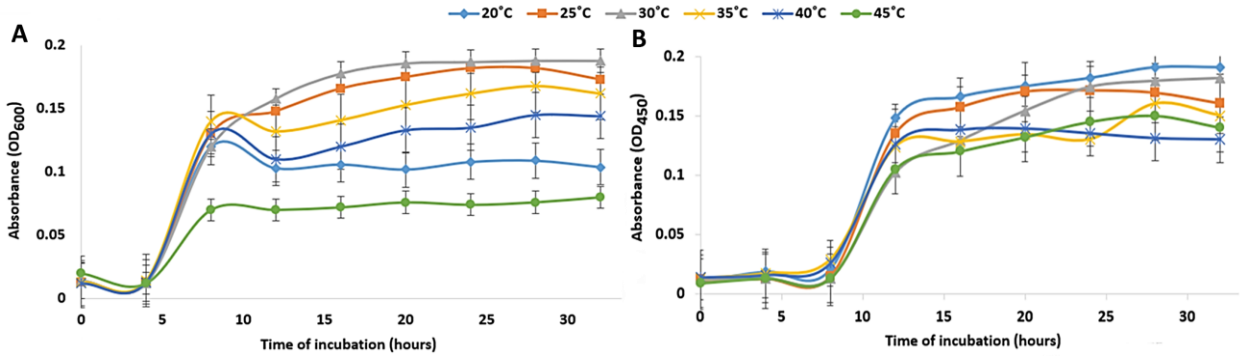

Fig. 1 Growth curve of *Kluyveromyces marxianus* (**A**) and *Ceratocystis fimbriata* (**B**) determined at different temperature values in liquid culture.

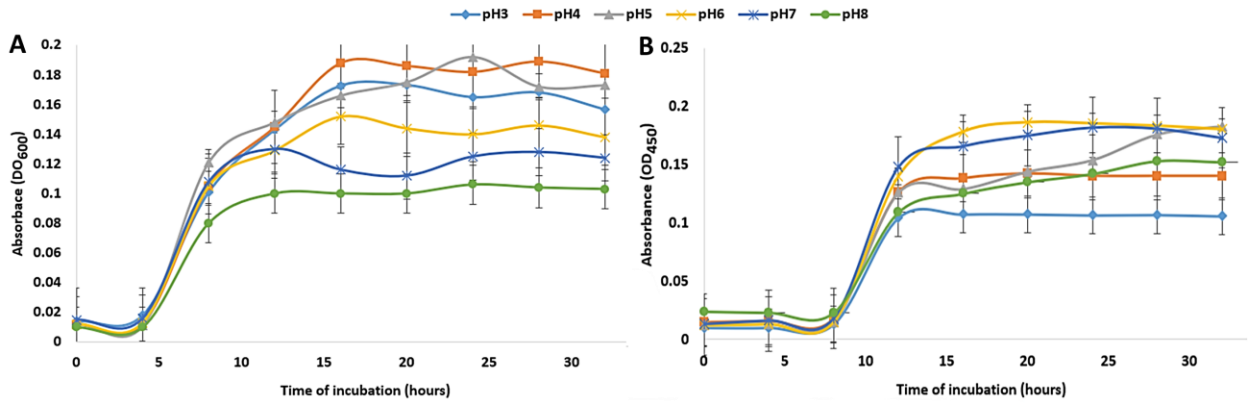

Fig. 2 Growth curve of *Kluyveromyces marxianus* (**A**) and *Ceratocystis fimbriata* (**B**) determined at different pH values in liquid culture.

Table 1 Analysis of variance for the effect of different temperature and pH levels at different time intervals on the growth of *Kluyveromyces marxianus* and *Ceratocystis fimbriata*.

Parameters	*Kluyveromyces marxianus*		*Ceratocystis fimbriata*	
	***F* value**	***P* value**	***F* value**	***P* value**
Temperature	1681.89	<0.0000	303.68	<0.0000
Time	3924.99	<0.0000	6770.69	<0.0000
pH	72.55	<0.0000	537.41	<0.0000
Time	478.91	<0.0000	5484.54	<0.0000

incubation. On the other hand, microbial culture *C. fimbriata* showed a bit longer initial lag phase and the microbial culture entered the exponential phase after 8 hrs of incubation, and the optimum growth was observed at 20˚C.

Microbial growth in different pH values

The influence of different pH levels on the growth of *K. marxianus* and *C. fimbriata* is shown in Fig. 2. The average optimum growth of the yeast *K. marxianus* was noted at pH between 4 to 5 with OD_{600} of 0.191, it was again observed that microbial culture enters an exponential growth after 4 hrs of incubation. Hence, pH 4.5 can be considered a suitable pH for *K. marxianus* for growth. *C. fimbriata*, on the other hand, showed average optimum growth at neutral pH between 6-7 with OD_{450} of 0.185. The microbial culture *C. fimbriata* again showed a bit longer lag phase in the growth curve as it entered the log (exponential) phase after 8 hrs of incubation. At pH 6 and 7, It reached to stationary phase after 14 hrs of incubation. Hence, pH 6.5 can be considered a suitable pH for *C. fimbriata* for growth.

Discussion

In this comparative study, the physiological growth of *K. marxianus* and *C. fimbriata* at different levels of temperature and pH was assessed *in-vitro* in potato dextrose broth. Physiological growth was measured based on the turbidity of the culture broth media. Initially, the culture media was clear hence the absorbance was 0.01, gradually the turbidity of the broth increased with increasing time of incubation leading to an increase in the absorbance. As both microorganisms produce bio-flavors, this study was conducted for their industry relevance [3]. According to this study, it was observed that yeast *K. marxianus* can survive at a wide range of temperatures from 20-45˚C but showed optimum growth at 30˚C while the optimum growth of *C. fimbriata* was observed at 20˚C. The optimum production of secondary metabolites such as ethanol production rages between 30-35˚C [16]. A recent study reported similar results that 23˚C was the optimum growth temperature for *C. fimbriata* [14]. *K. marxianus* reaches the highest growth at pH 4.5, while *C. fimbriata* showed optimum growth at pH between 6-7. In a previous study, a similar pH range from 3.5 to 4.5 was reported to be suitable for *K. marxianus* growth [17]. Similarly, a recent study supported our result showing the maximum growth of *C. fimbriata* in pH 7.5 on solid media [11]. The microorganism used for sustainable bio-flavors production should be able to grow in waste like fruit peel or pulp, which remains slightly acidic. Thus, choosing a microbial culture that can grow and able to produce secondary metabolites at acidic pH is better suitable for industrial use. Hence *K. marxianus* meet this aspect more than *C. fimbriata.* Furthermore, *K. marxianus* is better suitable for industrial bio-flavor production as it can sustain a wide range of temperatures during industrial fermentation. In addition. *C. fimbriata* reported to cause infection to angiosperms through roots and crown [18], thus it is not suitable for industrial use regarding environmental safety concerns. Hence, it is concluded that the fungal yeast *K. marxianus* is a better choice for industrial bio-flavor production due to its shorter lag and longer stationary phase, as well as its environmentally friendly nature and low downstream processing cost.

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Conflict of interest

The authors claim no conflicts of interest.

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