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Biotransformation of Flavonoid Naringin, Optimization of Various Fermentation Parameters

Vidya R. Kathoke[1] **and Gangadhar B. Shinde**[2]
Dharampeth & M. P. Deo Memorial Science College,Nagpur.
mail-vidyakatoke@yahoo.com

Abstract:

Microbial degradation of naringin a major bitter principle of citrus fruits into a nonbitter metabolite was studied by using indigenously isolated molds. Several efficient molds such as Aspergillus niger ITCC4515, Aspergillus flavus ITCC 4516, Aspergillus fumigatus ITCC 4517, Aspergillus terricola ITCC 4518, Penicillium nalgiovensis ITCC 4519 were isolated. The optimization of various fermentation parameters was performed. Percentnaringin degradation by each mold was determined by adjusting all the optimized conditions singly and in combination. Naringin degradation was found to be increased further in latter case when compared with former one. Degradation after adjusting all the optimized conditions in combination was maximum in Aspergillus niger90%, followed, in decreasing order, by Penicillium nalgiovensis 86.98%, Aspergillus terricola 85%, Aspergillus fumigates 83.98% Aspergillus flavus80%.

Keywords:
Flavonoid Naringin, bitterness, molds, degradation, grape fruit.

Introduction:

Naringin, a major bitter principle of grapefruit(5,6), is a flavonoid found principally in citrus fruits and affects the quality of citrus fruits and their products in a distinct way(2).Presence of flavonoid in in the flowers of grape plant was first discovered by DeVry(4). Naringin accumulates in appreciable amounts in grapefruit during fruit development and imparts immediate bitterness to the processed juice causing unpalatability(6) and thus reduces the export market potential of grapefruit and its products. Its bitter taste remains distinctly detectable in fresh, refrigerated, pasteurized, canned and frozen juices(9).

Because of bitterness problem, grapefruit based industries are finding difficulties in easy marketing of products both in local as well as international markets. Under this situation of serious economic threat to grapefruit processing industries, microorganisms may offer a concrete, economic and easy to operate solution to handle the bitterness problem (11,12). In view of the above facts, it was decided to undertake a more systematic study to convert a bitter flavonoid naringin to its nonbitter metabolite by using indigenously isolated microorganism.

Microorganisms from different generic category show different metabolic activity in different physical (pH, temperature, aeration, agitation) and chemical environments. Present study was therefore aimed mainly at

optimizing various physical and chemical fermentation parameters to get maximum naringin transformation.

Material and methods:

1. Microorganisms: Following indigenously isolated microorganisms were selected for present study since they were most promising in utilizing naringin as the sole carbon source at acidic pH. *Aspergillus niger* ITCC-4515, *Aspergillus flavus* ITCC4516, *Aspergillus fumigatus* ITCC4517, *Aspergillus terricola* ITCC4518 and *Penicillium nalgiovensis* ITCC4519,

2.Standard naringin,

3. Nitrogensources: Ammonium sulphate, Sodium nitrate, Ammonium chloride and Ammonium nitrate.

4. Growth factors: CSL, YE, Vitamin B-complex,

5. Davis reagent(3)**:** (Alkalinediethylene glycol),

6. Medium: Mineral medium Saunders et al., (8).

Series of 50 ml flasks each containing 25ml of Saunders medium with exogenously added naringin 0.1% used for studying naringin degradation by microorganisms under different fermentation conditions. A common uninoculated control was run in parallel for each parameter.

Preparation of Inoculum: A series of tubes containing 5ml distilled water, sterilized and five different microbial suspensions prepared by suspending aseptically two-three loopfuls of 48-72hrs old mould cultures. Tubes were kept on mechanical shaker to evenly distribute cells. Homogeneous culture suspensions were separately used as an inoculum for acclimatization set.

Acclimatization: Since, mold cultures maintained on CzapekDox agar slants, it was felt necessary to activate its naringin utilizing capacity by separately incubating them for 48hrs in Saunders medium containing low level naringin(0.02%) as a sole carbon source. Molds thus, acclimatized to use naringin in the medium as follows:

A series of test tubes each containing 10ml Saunders medium (pH-4.5)with 0.02%naringin was sterilized and inoculated separately with homogenous mold culture suspensions(5ml)and incubated at room temp (35±3.5˚C) for 48hrs. Incubation mixture from each tube was filtered. The acclimatized cells (biomass) thus obtained were washed with sterilized naringin free Saunders medium and used for final optimization sets.

Effect of pH: A set of six flasks, containing 25ml Saunders medium with naringin-0.1%, pH-3.5 were sterilized. Acclimatized cell biomass of molds separately inoculated in the medium of five flasks. Sixth flask was kept as uninoculated control and incubated at room temperature for 96hrs with constant agitation on shaker at 280rpm.Since experiment was aimed at three different pH values, similar procedure was repeated for studying naringin degradation at pH-4.5 and 5.5.

Effect of Temperature: In an attempt to explore the possibility that temperature might affect metabolic activity of an organism, naringin

degradation capacities of molds were studied at 28,30and37˚C.Similar experimental procedures like pH were carried out. Flasks incubated at desired temperatures for 96hrs with constant agitation at 280rpm in an incubator (Widson Scientific Works, New Delhi, India) provided *in situ* with mechanical shaker.

Effect of Nitrogen Sources: Microorganisms differ in their metabolic activity in different chemical environments, hence a study on effect of various N-sources on naringin degradation by molds was conducted. For this, a nitrogen free Saunders medium was prepared (8) excluding N-sources like ammonium nitrate, sodium nitrate and ammonium phosphor molibdate. Preferred N-sources were ammonium sulphate, ammonium chloride, ammonium nitrate and sodium nitrate. Amount of each of these N-sources were calculated to give total final concentration of 0.84gN/lit of the medium (total nitrogen as in Saunders medium) added exogenously. The experimental procedure were same as that used for pH and temp. An experimental control set(nitrogen free)was run.

Effect of Growth Factors: Since microorganisms differ in its capacity to utilize different growth factors, experiments were designed to study effect of CSL,YEand vitamin B-complex on naringin degradation. Three different concentrations for CSL and YE were 0.2%, 0.5%, 1.0% (w/v); and vitamin Bcomplex- 0.01%, 0.05%, 0.1% (w/v). Same experimental procedure as used to study effect of N-sources were repeated, along with experimental controls.

Effect of Aeration: To study the effect of aeration, during incubation, sterilized air was bubbled at a rate of 0.5vvm through medium. A set of six flasks prepared and treated in the same manner except no air was bubbled(nonaerated set).

Effect of Optimized Parameters in Combination: This experiment was designed to study effect of combined optimized parameters like pH, temperature, N-sources, growth factors and aeration on rate of naringin transformation by molds (Table-4).

Assay of Residual Naringin: Five ml aliquots of culture filtrate were removed aseptically from each flasks of above sets after every 24 hrs incubation, filtered with membrane filter. Clear culture filtrates separately analysed by Davis method (3). Percent transformation of naringin calculated by comparing experimental values with respective uninoculated controls.

Results and discussion:

Microorganisms differ in regard to their metabolic activity in different physical (pH, temperature, aeration, agitation)and chemical(N-sources, growth factors) environments. Results on effect of individual optimized fermentation conditions on degradation of naringin by five molds are presented in Tables1-3.

Result and discussion:

Effect of pH: In order to promote industrial use of such study, it becomes necessary to perform experiment under as natural environment as possible.

Native pH of grapefruit is acidic (4.5±0.75) depending upon ripening stage of fruit at the time of processing. In this context three pH values selected viz. pH-3.5, 4.5 and 5.5 for naringin transformation studies.

Results showing effect of optimized pH on naringin degradation by molds are given in Table-1. *A. niger* and *A. fumigatus* showed respectively 50%and45% transformation at pH-4.5; whereas, *A. flavus, A. terricola* and *P. nalgiovensis* showed maximum degradation at pH-5.5, as 43%,47% and 45% after 96hrs. Transformation at pH-3.5 was consistently poor. Based on results of naringin degradation, at pH-4.5 considered an optimum for *A. niger*and *A. fumigatus;* pH- 5.5 optimum for *A. flavus, A. terricola, P. nalgiovensis.*

Effect of Temperature: It is well established that microorganisms are highly active at 28˚C (molds), 37˚C (bacteria). Hence, naringin degradation was carried out at three temperatures 28˚C, 30˚C and 37˚C. Table-1, shows effect of temperature on naringin degradation by five molds. After 96hrs it was between 35.55%-43.85% at 28˚C; 41.55%-46.95% at 30˚C; and 45%-50% at 37˚C.

Effect of Nitrogen Sources: Fact that microorganisms differ in its metabolic activity in different chemical environments was confirmed by studying effect of different inorganic N-sources singly on naringin degradation capacity of organisms. N-sources selected were ammonium sulphate, ammo. chloride, sodium nitrate and ammonium nitrate. Naringin transformation using preferred N-sources are presented in Table-2 Of the N-sources tried, ammonium nitrate was found to be best for naringin degradation by *A. niger* and *A. fumigates* showing maximum degradation values 57.44% and 52.40% respectively within 96 hrs, when compared with experimental control set. Whereas, ammo. sulphate was found to be the best N-source for *A. flavus, A. terricola* and *P. nalgiovensis* showing degradation values 50%,55% and 54% within 96 hrs. As evident from Table-2 ammo.sulphate can be considered as the most preferred N-source for *A. flavus, A. terricola* and *P. nalgiovensis;* ammo. Nitrate for *A. niger* and *A. fumigatus.* Similar studies carried out by Smythe, Moorestown and Dudley (9) where they used ammonium phosphate as preferred N-source to obtain maximum biomass of naringinase producing strains. Another studies carried out by Thammawat K., *et al.,* (10) highest naringinase activity was obtained with $NaNO_3$ (2.5g/lit) as preferred N-source. From above observations it can be concluded that presence of different N-sources in the medium affects naringin degradation ability of molds when compared with nitrogen free sets.

Effect of Growth Factors: Effect of different growth factors on naringin degradation capacity of molds were studied. As evident from the Table-3 maximum naringin transformation was observed in 0.2%CSL as compared to control tubes (without CSL). After 96hrs naringin degradation rate shown by five molds was, *A. niger* 46%-65%, *A. flavus* 40% - 58%, *A. fumigates* 43%-60%, *A. terricola* 46%-62% and *Penicillium nalgiovensis* 44%-60%. However, percent transformation by five molds in medium containing 0.5% and 1.0%

CSL was appreciably lower than at 0.2 %CSL. Naringin degradation at 0.05 % vitamin B-complex was higher than at 0.01 % and 0.1 % levels. When results of degradation at 0.05 % vitamin B-complex were compared with experimental control, after 96hrs were found to be increased from 46 %-62 % in *A. niger,* 40 %-51 % in *A. flavus,* 43 %-55 % in *A. fumigatus,* 46 %-56 % *A. terricola* and 44 % - 55 % *P. nalgiovensis* (Table-3).

Similarly most preferred level of YE in the medium for maximum naringin degradation was found to be 0.2 %, whereas degradation values at 0.5 % and 1.0 %YE were comparatively lower. Transformation of naringin increased at 0.2 % YE in *A. niger* from 46 % - 55 %, *A. flavus* 40%- 49%, *A. fumigates* 43% - 53%, *A. terricola* 46 % - 53 %, *P. nalgiovensis* 44 % - 49 % after 96 hrs against respective experimental controls. Unlike CSL, YE at higher concentration resulted in lower rate of naringin transformation (Table-3).

The molds showed maximum transformation of naringin in presence of 0.2% CSL, followed by 0.05%Vit.B-complex and lastly by 0.2% YE. The use of CSL was found to be advantageous over vitamin B-complex and YE because of the following reasons-its low carbohydrate content, easy to handle, reduced sterilization difficulties especially when used on a large scale as suggested by Bram and Solomons (1). Puri et al., (7) reported use of molasses as carbon source in their experiment.

Effect of Aeration: Table-1 shows effect of aeration on naringin transformation by molds. During estimation of residual naringin after every 24hrs, it was observed that aerated flasks showed more naringin trans-formation as compared to nonaerated flasks. As evident from Table, all molds showed 40%-45% naringin degradation within 96hrs in nonaerated flasks, while in aerated flasks degradation was in the range of 43%-50%.

Combined Effect of Optimized Fermentation Conditions: Experiments were designed to see whether or not all optimized fermentation conditions shown inTable-1-3, when performed in combination all at a time would augment rate of transformation any further. Data of combined effect of optimized parameters onnaringin transformation by molds presented in Table-4. As shown in Table percent transformation of naringin was increased further from 50 %-65 %to 90 % in A. niger; 43 %-58 % to 80 % in A. flavus; 45 %-60 % to 84 % in A. *fumigatus*; 47 %-62 % to 85 % in A. terricola; 45 %-60 % to 87 % in P. nalgiovensis after 96 hrs. Thus, molds, especially Aspergillus and Penicillium species, can be considered as promising candidates for transformation of naringin a major bitter principle in citrus fruit products.

Table–1: Effect of pH*and temperature**and aeration*** on percent transformation (%T)of naringin by five molds in Saunders medium

Micro-organisms	Time hrs	Aeration % T		pH % T			Temp % T		
		No-air	Aerated	3.5	4.5	5.5	28˚C	30˚C	37˚C
A.niger	96	45.20	49.85	44.95	50	47.45	40.25	46.95	50
A.flavus	96	39.95	42.70	32.95	38	42.64	35.55	41.55	45.00
A.fumigatus	96	43.95	48.40	41.55	45	44.85	43.85	44.35	46.95
A.terricola	96	44.98	48.80	37.90	41.55	46.95	38.00	45.00	47.45
P.nalgiovensis	96	43.95	47.45	31.85	41.55	45.00	36.70	42.70	49.78

Each Value is mean of three replicates, naringin - 0.1g/ 100ml
***pH-4.5± 0.5,**Flasks incubated at RT-35±3.5˚C,agitation280rpm.**
%T calculated by comparing experimental value with its respective control.*Sterilized air bubbled through flask.**

Table-2: Effect of different nitrogen sources on percent transformation (%T) of naringin by molds in Saunders medium

Micro-organisms	Incubation time(hrs)	Nitrogen free	Preferred N-sources			
		medium	Ammo sulphate	Ammo nitrate	Ammo chloride	Sodium nitrate
		(%T)	(%T)	(%T)	(%T)	(%T)
A.niger	96	15	52.40	57.44	52.40	50
A.flavus	96	12.40	49.87	45	41.95	40
A.fumigatus	96	15.98	50	52.40	43.60	48.70
A.terricola	96	12.40	54.80	50	45	47.40
p.nalgiovensis	96	15	52.40	57.44	52.40	50

Each Value is mean of three replicates.Naringin-0.1g/ 100ml Flasks incubated at RT-35±3.5˚C,agitation280rpm, pH-4.5±0.5.%T calculated by comparing experimental value with its respective control values

Table–3: Effect of different concentrations of growth factors on percent transformation(%T)of naringin by molds in Saunders medium

		wihout	Growth factors								
		Growth	CSL (%T)			Vitamin B-complex (%T)			YE (%T)		
Micro-organisms	Time (hr)	Factor (%T)	0.2%	0.5%	1.0%	0.01%	0.05%	0.1%	0.2%	0.5%	1.0%
A.niger	96	45.55	64.75	56.00	49.30	55.00	61.90	48.80	55.00	50.95	50.95
A.flavus	96	39.85	58.00	45.55	42.00	46.66	51.00	43.00	49.00	41.98	40.00
A.fumigatus	96	43.00	60.00	53.00	49.98	50.95	55.00	48.80	53.00	48.80	50.95
A.terricola	96	45.55	62.00	47.75	45.55	51.00	56.00	46.66	53.00	48.80	49.00
P.nalgiovensis	96	44.35	60.00	49.00	42.00	46.66	55.00	40.00	49.00	40.00	40.00

Each value is mean of three replicates,Naringin-0.1g/ 100ml Flasks incubated at RT-35±3.5 ˚C,agitation280rpm,pH-4.5±0.5 %T calculated by comparing experimental value with its respective control.

Table–4: Effect of combined optimized fermentation conditions on percenttransformation(%T)of naringin by five molds in Saunders medium

Microorganisms	Incubation Time(hrs)	Saunders med. without any parameters(%T)	Saunders med.with combined* optimized fermentation parameters(%T)
A.niger	96	45.00	90.00
A.flavus	96	39.98	80.00
A.fumigatus	96	43.98	83.98
A.terricola	96	45.00	85.00
p.nalgiovensis	96	44.80	86.98

Each Value is mean of three replicates,naringin-0.1g/100ml %T calculated by comparing experimental value with its respective control.Incubation at RT-35±3.5˚C,agitation280rpm, pH-4.5±0.5.*All optimized conditions were adjusted in combination all at a time for this set.

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