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A Study on the Development of Talaromycesflavus Formulations by a Fermenter and Some of their Biological Properties

LALEH NARAGHI[1*], SHAHRAM NAEIMI[1], RASOUL MARZBAN[1], ASGHAR HEYDARI[1] AND MOHAMMAD RAZAVI[1]

[1]*Iranian Research Institute of Plant Protection, Education and Extension Organization (AREEO), Tehran, Iran.*

**Corresponding Author E-mail: lale_naraghi@yahoo.com*

Abstract.
In Iran, a large body of research has demonstrated the importance of the antagonist fungus, *Talaromycesflavus*, in inhibiting the growth of some important soil-borne pathogensuch as *Rhizoctoniasolani*, *Verticilliumdahlia*, and *Fusariumoxysporum*. According to results obtained up to this stage of studies, commercialization of this fungus bioformulationis of particular importance. Since marketing is considered to be an important factor for continued commercialization, paying attention to bioformulationtype with eveneasier application, low consumption, and an appearance similar to common chemical fungicides can be largely influential in attracting relevant consumers and having a successful marketing. To continue the above research, preparations were made from different bioformulations of the aforesaid biological agent with an emphasis on facilitating the application and possibly with similar formulations of chemical fungicides available in the market to investigate their efficacy in biological control of plant disease. Eight liquid bioformulations was prepared from *T. flavus* using a fermenter by manipulation of several important factors, namely two different temperatures, two types of media, and two types of stabilizers. Among the eight prepared liquid bioformulations, the most effective ones were selected for greenhouse evaluation in terms of the lowest infection percentage, maximum sporulation and active population, and the highest mycelial mass. These bioformulations were CDB + NaNO3 + 30 °C, CDB + NaNO3 + 25 °C, CDB + D-cycloserine+ 30 °C, and PDA + D-cycloserine+ 25 °C. According to results of greenhouse study on four formulations with two different application methods (addition to soil and seed impregnation), two treatments of CDB + NaNO3 + 25 °C and PDA + D-cycloserine+ 25 °C with seed impregnation method were the most effective treatments in increasing the number of healthy sugar beet plants.

Keywords. Sporulation, Stability, Liquid Formulation, *Talaromycesflavus*

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INTRODUCTION

In Iran, recent studies have reported favorable results concerning the antagonist fungus *T.flavus* for the control of some important soil-borne pathogens such as *Verticilliumdahliae, V.albo-atrum, Fusariumoxysporum*, and *Rhizoctoniasolani* in some crops including cotton, sugar beet, potato, tomato, and greenhouse cucumbers (Naraghi et al., 2010a; Naraghi et al., 2010b; Naraghi et al., 2010c; Naraghi et al., 2012a; Naraghi et al., 2012b). In addition, the application of this fungus in the fields as proliferatedin the state ofsolid fermentation on plant residues or their mixture with peat soil led to reduced disease incidence rate and increased yields in the above crops. Naraghi et al. (2014b) observed 50% reduction in *Verticillium* wilt disease, 37% reduction in seedling death rate, 30% yield increase in cotton, 40% decrease in disease percentage, 17% increase in potato yield, 93 % increase in healthy seedlings, and 50% increase in sugar beetyield. In tomato, 27% decrease in disease severity, and 23% increase in yield, and 30% drop in disease severity and 7% performance increase in greenhouse cucumber were detected by Farhang Niya et al. (2015).

Marketing and attracting relevant consumers account for important issues in the commercialization of a biological product (Alimi et al., 2006; Husen et al., 2007; Kaewchai et al., 2009; Pereira et al., 2009); therefore, research is needed on the production of optimal bioformulations with increasing efficiency, easier consumption, more cost-effectiveness, and better appearance. Among various bioformulations of fertilizers and biological and chemical products, liquid bioformulation has received a particular boom in the market (Haggag et al., 2013; Shahid et al., 2014); these bioformulations have been of great interest due to their applicability to water in irrigation systems. A combination of these two factors (water and fertilizer) or so-called fertilization with irrigation (fertigation) has particular advantages. Success in this method requires sufficient knowledge of both the water and fertilizer requirements of any crop. This methodminimizes environmental pollution due to optimum use of water and fertilizer, and fertilization is easily fractioned in sensitive and required stages of the plant. It also prevents the loss of fertilizer due to the control of soil nutrient contents, which is supplied to the plant depending on its growth requirements. Additionally, it is better absorbed due to the uniform solubility of fertilizers in irrigation water (Russo, 2006).

Given the importance of proper water use and optimum water efficiency, achieving the goals of irrigation systems in agriculture has been prioritized in the country.A goal of these systems is to apply chemical and biological fertilizers and pesticides together with irrigation water indrip and sprinkler systems. Injection of fertilizer to irrigation water involves dissolving water-soluble fertilizers and applying them through irrigation systems. It also reduces fertilization costs by excluding operations, and slowly increases root density and length (Khelil et al., 2005; Wszelaczynska et al., 2015; Dunn et al., 2016). Therefore, due to the efficiency of *T.flavus* in increasing yield and boosting vegetative traits (Naraghi et al., 2012a; Naraghi et al., 2012b; Naraghi et al., 2014a; Naraghi et al., 2014b), the preparation of liquid bioformulation from this fungus can not only facilitate the

marketing, but also play the role of liquid fertilizer and its application by fertilizer injection to irrigation water resulting in the same benefits as fertilization with irrigation.

Previous research has shown that enrichment of liquid bioformulations with effective antagonistic fungal effective metabolites to be very efficient in enhancing the biocontrol efficiency of these bioformulations. It can, therefore, be acknowledged that increasing the stability of metabolites in bioformulations also has an effect on raising their biocontrol ability. According to previous studies on increasing the stability of *T. flavus* metabolites by the use of chemical stabilizers, including dicycloserine and NaNO3, a liquid bioformulation of *T. flavus* was prepared and enriched with the above stabilizers to evaluate its biocontrol ability for some important plant diseases.

MATERIALS AND METHODS

Preparation of T. flavus liquid formulation with a fermenter application

T. flavus liquid formulation was prepared using a 4-L fermenter available at the Biological Control Department of the Iranian Research Institute of Plant Protection, based on the method of Mascarin et al. (2015). Two culture media, including starter medium and fermentation medium, were prepared in autoclavable propylene jar-like vessels with a volume of 4 L and a mouth diameter of 4 cm. The antagonist fungus was inoculated into the starter medium and was finally used to inoculate into the fermentation medium.

According to the above method, 400 ml of the starting medium and 3 L of the fermentation medium were used for a volume of 4 L. A singletype of liquid medium was used for both types of media, and 30 g of molasses and 5 g of yeast were used per 1.0 liter. To prepare the starting medium, half of the desired volume (200 ml of 400 ml) was inoculated with the antagonist fungus at a concentration of 10^9cfu/ml. To reach this concentration, a medium suspension was prepared at a concentration of 4×10^{11}ml, of which1.0 ml was adjusted to 200 ml together with the medium and placed on a shaker for 5 days. To prepare the starting medium with a volume of 400 ml, the culture medium (200 ml) was added to this suspension. Finally, the starting culture medium (400 ml) was inoculated into fermentation medium (3 L) and placed in a fermenter apparatus to produce a liquid ioformulation with a volume of 400 L.

The shaking rate and time of the fermenter was set properly using the most appropriate time and shaking rate in terms of spore count/ml (cfu) according to results of the preliminary stage. The fermentation medium pH was set at 8 based on the optimum pH for *T. flavus* mass production (Marois et

al., 1984). Antifoam additives were also used based on previous investigations (Papavizas et al., 1984; Hebbar et al., 1997; Viccini et al., 2007; Kolombet et al., 2008).

Different treatments were applied for the preparation of liquid formulation, including temperatures (25 and 30 °C) governing the fermentation process (Marois et al., 1984; Kolombet et al., 2008), the type of liquid media*viz*. Czapek Dextrose Broth and Potato Dextrose Broth (Khan et al., 2011), and two types of stabilizers namelyNaNO3 and D-cycloserine. Accordingly, eight different treatments were obtained based on the above variable factors used in the fermentation process (Figure 1).

Figure 1. Liquid formulations prepared based on the media (PDA and CDB), temperature (25 and 30 °C), and stabilizers (NaNO3 and D-cycloserine)

Evaluation of mycelial mass, sporulation, active population, and infection percentage in prepared liquid bio-formulations

At this stage, first the mycelial mass of different bio-formulations was measured at the production time. To calculate the mycelial mass, 40 ml of the 72-h sample was centrifuged at 10,000 rpm for 20 min (Figure 2). The supernatant was discarded, the precipitate was placed in an oven at 50 °C for 2-3 days, and its weight was determined after drying (Sutton and Starzyk, 1972).

Figure 2. Centrifugation of *T.flavus* liquid formulations to determine mycelial mass weight

Then, the sporulation rate (The colony-forming units per milliliter or CFU/mil), active population (shelf life), and infection (%) of liquid bio-formulations were evaluated at storage conditions at 25 and 4 °C three months after production and continued until fifteen months post-production with three-month intervals.

RESULTS

Mycelial mass weight of fermenter-produced T. flavus liquid formulations

The test for mycelial mass weight of fermenter-produced *T. flavus* liquid formulations was significant at 1% level. The results of mean mycelial mass in different formulations determined five statistical groups for the formulations (Table 1).

Maximum weights of mycelial mass were recorded in liquid formulations ofLF-1 (CDB + 30 °C + NaNO3), LF-2 (CDB + 25 °C + NaNO3), LF-3 (CDB + 30 °C + D-cycloserine), and LF-8 (PDB + 25 °C + D-cycloserine) (Table 1). LF-5 (PDB + 30 °C + NaNO3) and LF-7 (PDB + 30 °C + D-cycloserine) formulations presented the lowest mycelial mass weights (Table 1). Also, no significant differences were foundbetweenLF-3 (CDB + 30 °C + D-cycloserine) and LF-4 (CDB + 25 °C + D-cycloserine) formulations in terms of mycelial mass weight (Table 1).

Table 1. Comparison of mean mycelial mass weight in 40 ml of different *T. flavus* liquid formulations after production in thefermenter

T. flavus liquid formulations (LF*)	Mycelial mass weight (g)
LF-1 (CDB + 30 °C + NaNO3)**	0.59c
LF-2 (CDB + 25 °C + NaNO3)	0.80b
LF-3 (CDB + 30 °C + D-cycloserine)	0.56c
LF-4 (CDB + 25 °C + D-cycloserine)	0.52cd
*** LF-5 (PDA + NaNO3 + 30 ºC)	0.35e
LF-6 (PDA + NaNO3 + 25 ºC)	0.48d
LF-7 (PDA + D-cycloserine + 30 ºC)	0.39e
LF-8 (PDA + D-cycloserine + 25 ºC)	0.94a

*LF: Liquid Formulation, **CDB: Czapeck Dextrose Broth, ***PDA: Potato Dextrose Broth

• There are no statistically significant differences between the means with similar letters at 1% level.

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Investigating the sporulation rate of T. flavus liquid formulations within 15 months post-production at 25 °C

The sporulation test of *T. flavus* liquid formulations within 15 months post-production at 25 °Cwas significant at 1% level, while it was not significant at the fifth trimester. The results of this experiment showed an increased sporulation in all formulations (spore count per ml of liquid formulation logarithm) from three months (first trimester) to six months (second trimester) after production (Table 3). In the third trimester, this level decreased in all formulations but it increased in LF-1, LF-5, LF-6, and LF-8 formulations only in the fourth trimester (Table 2).

In the first to the fourth trimesters after production, the obtained results indicated that the highest sporulation rate belonged to LF-1 (CDB + 30 °C + NaNO3), LF-2 (CDB + 25 °C + NaNO3), LF-3 (CDB + 30 °C + D-cycloserine), and LF-8 (PDB + 25 °C + D-cycloserine) liquid formulations (Table 2).

Table 2. Comparison of sporulation mean per 1.0 ml of different *T. flavus* liquid formulations at 25°C

T. flavus liquid formulations	First trimester		Second trimester		Third trimester		Fourth trimester		Fifth trimester	
	Logarithmic spore count	Spore count/ml ($\times 10^9$)	Logarithmic spore count	Spore count/ml ($\times 10^9$)	Logarithmic spore count	Spore count/ml ($\times 10^9$)	Logarithmic spore count	Spore count/ml ($\times 10^9$)	Logarithmic spore count	Spore count/ml ($\times 10^9$)
LF-1 (CDB + 30 °C + NaNO3)	7·698f	0·050	8·698b	0·500	8·203ab	0·160	8·217b	0·165	7·371a	0·025
LF-2 (CDB + 25 °C + NaNO3)	7·698f	0·050	8·932a	0·872	8·267a	0·187	8·217b	0·165	7·397a	0·025
LF-3 (CDB + 30 °C + D-cycloserine)	8·291c	0.200	8·704b	0·512	8·221a	0·167	7.901bc	0·080	7·541a	0·020

LF-4 (CDB + 25 °C + D-cycloserine)	8·590b	0·390	7·861cd	0·075	8·044b	0·112	7·378c	0·010	7·301a	0·020
LF-5 (PDA + NaNO3 + 30 ºC)	9·070a	1·180	7·473f	0·030	7·079e	0·012	7·874bc	0·075	7·300a	0·020
LF-6 (PDA + NaNO3 + 25 ºC)	8·072d	0·112	7·698d	0·050	7·563d	0·037	7·845bc	0·070	7·392a	0·025
LF-7 (PDA + D-cycloserine + 30 ºC)	8·175cd	0·150	7·170e	0·015	7·735c	0·055	7·634c	0·050	7·397a	0·025
LF-8 (PDA + D-cycloserine + 25 ºC)	7·864e	0·075	7·903c	0·080	7·872c	0·075	8·733a	0·100	8·446a	0·025

*LF: Liquid Formulation, **CDB: Czapeck Dextrose Broth, ***PDA: Potato Dextrose Broth

• There are no statistically significant differences between the means with similar letters at 1% level.

Investigating sporulation rate of T. flavusliquid formulations within a 15-month period after production at 4 °C (refrigerator)

The spore determination test of *T. flavus* liquid formulations in a 15-monthpostproduction at 4°C was significant at 1% level. From three months (first trimester) to six months (second trimester) after production, there were decreases in spore count (number of spores per ml of logarithmic liquid formulation) in all formulations (Table 3). The spore reduction trend continued in the third trimester, with the exception of three formulations (LF-5, LF-6, and LF-7). This level had no changes in the fourth compared to the third trimesters, and decreased in all formulations in the fifth trimester (Table 3).

The results showed the best formulations were related to those containing CDB medium (LF-1, LF-2, LF-3, and LF-4) in the first trimester post-production (with maximum spore count; Table 5). In the fifth trimester

after production (with the lowest spore count), however, those containing PDB medium (LF-5, LF-6, LF-7, and LF-8) were the best formulations (Table 3).

Table 3. Comparison of average sporulation in one ml of different *T. flavus* liquid formulations at 4 °C

T. flavus liquid formulations	First trimester		Second trimester		Third trimester		Fourth trimester		Fifth trimester	
	Logarithmic spore count	Spore count/ml ($\times 10^9$)	Logarithmic spore count	Spore count/ml ($\times 10^9$)	Logarithmic spore count	Spore count/ml ($\times 10^9$)	Logarithmic spore count	Spore count/ml ($\times 10^9$)	Logarithmic spore count	Spore count/ml ($\times 10^9$)
LF-1 (CDB + 30 °C + NaNO3)	8·563b	0·375	7·553bc	0·032	7·096d	0·012	7·096c	0·165	6·778d	0·006
LF-2 (CDB + 25 °C + NaNO3)	8·653b	0·450	8·419bc	0·027	7·096d	0·012	7·096c	0·165	6·778d	0·006
LF-3 (CDB + 30 °C + D-cycloserine)	8·875a	0·750	7·693b	0·050	7·096d	0·012	7·096c	0·080	7·301c	0·020
LF-4 (CDB + 25 °C + D-cycloserine)	8·602b	0·400	8·096a	0·125	7·301c	0·020	7·301b	0·020	7·602a	0·040
LF-5 (PDA +	7·697c	0·050	7·563bc	0·037	7·759a	0·057	7·740a	0·055	7·511ab	0·032

NaNO3 + 30 ºC)										
LF-6 (PDA + NaNO3 + 25 ºC)	7·380d	0·024	7·601bc	0·040	7·698ab	0·050	7·698a	0·050	7·414bc	0·026
LF-7 (PDA + D-cycloserine + 30 ºC)	7·698cd	0·050	7·446bc	0·025	7·602b	0·040	7·602a	0·040	7·477ab	0·030
LF-8 (PDA + D-cycloserine + 25 ºC)	7·242e	0·017	7·242c	0·017	7·096d	0·012	7·096c	0·012	6·880d	0·007

*LF: Liquid Formulation, **CDB: Czapeck Dextrose Broth, ***PDA: Potato Dextrose Broth

• There are no statistically significant differences between the means with similar letters at 1% level.

Determination of the active population (stability) of T. flavus liquid formulations over a 15-month period after production at 25 °C

The determination of active population of *T. flavus* liquid formulations was significant at 1% level in a 15-month period after production at 25 °C. After examining the active population of *T. flavus*, green fungal colonies were observed with a bright yellow halo (Figure 3). In the first trimester post-production, infections of saprophytic fungi (*A.niger* and *Penicillium* spp.) were found considerably in LF-5 and LF-7 formulations resulting from mass production in the fermenter (Figure 3).

The results of this experiment showed that from three months (first trimester) to six months (second trimester) post-production, there were increases in the active population (per1.0 ml of logarithmic liquid formulation) in all formulations (Table 4). In the third trimester, however, this level decreased in all formulations except LF-8 (PDB + 25 °C + D-cycloserine) and continued to decrease in all formulations until the fifth trimester (Table 4). In LF-8 formulation, an increasing trend of active population was observed from the first to the third trimesters after production (Table 4).

In the first to the fifth trimesters after production, the highest active population belonged to liquid formulations of LF-1 (CDB + 30 °C + NaNO3), LF-2 (CDB + 25 °C + NaNO3), LF-3 (CDB + 30 °C + dicycloserine), and LF-8 (PDB + 25 °C + D-cycloserine) (Table 4).

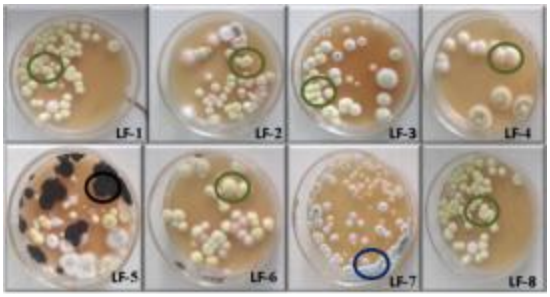

Figure 3. Investigating the active populations of *T.flavus*in liquid formulations (LF-1, LF-2, LF-3, LF-4, LF-5, LF-6, LF-7, and LF-8) in the first trimester post-production; colony of *T. flavus* (green circle), and infections with *Aspergillus niger* (clack circle) and *Penicillium* spp. (blue circle)

Table 4. Comparison of active population in 1.0 ml of T. flavus liquid formulations at 25 °C

T. flavus liquid formulations	First trimester		Second trimester		Third trimester		Fourth trimester		Fifth trimester	
	Logarithmic spore count	Spore count/ml (× 10^9)	Logarithmic spore count	Spore count/ml (× 10^9)	Logarithmic spore count	Spore count/ml (× 10^9)	Logarithmic spore count	Spore count/ml (× 10^9)	Logarithmic spore count	Spore count/ml (× 10^9)
LF-1 (CDB + 30 °C + NaNO3)	7·698b	0·050	8·698b	0·500	8·204a	0·160	7·217a	0·0165	6·397b	0·0025
LF-2 (CDB + 25 °C + NaNO3)	7·698b	0·050	8·940a	0·872	8·271a	0·187	7·217a	0·0165	6·397b	0·0025
LF-3 (CDB + 30 °C + D-	7·096c	0·012	8·709b	0·512	8·222a	0·167	7·204a	0·0160	6·602ab	0·004

cycloserine)										
LF-4 (CDB + 25 °C + D-cycloserine)	7·698b	0·050	7·527c	0·033	6·892c	0·007	5·000d	0·0001	5·301c	0·0002
LF-5 (PDA + NaNO3 + 30 ºC)	8·549a	0·354	7·269de	0·018	5·924d	0·0008	5·875c	·000750	5·301d	0·0002
LF-6 (PDA + NaNO3 + 25 ºC)	7·653b	0·045	7·352cd	0·022	5·574f	0·0002	5·845c	0·0007	5·397c	·000250
LF-7 (PDA + D-cycloserine + 30 ºC)	7·667b	0·046	7·176e	0·015	5·740e	0·0005	6·000c	0·001	5·397c	·000250
LF-8 (PDA + D-cycloserine + 25 ºC)	7·673b	0·047	7·471c	0·029	7·875b	0·075	7·000b	0·01	6·698a	0·005

* LF: Liquid Formulation, ** CDB: Czapeck Dextrose Broth, *** PDA: Potato Dextrose Broth

• There is no statistically significant difference between means with similar letters at 1% probability level.

Determination of the active population (stability) of T. flavus liquid formulations over a 15-month period after production at 4 °C (refrigerator)

The determination of active population of *T. flavus* liquid formulations was significant at 1% level in a 12-month period after production at 4 °C. In the study of *T. flavus* active population, the fungal colonies were consistently green and in some cases white with a bright yellow halo (Figure 4). In the second trimester of production, bacterial and fungal (*A.niger*) infections were considerably observed in LF-6 and LF-7 formulations resulting from mass production in the fermenter (Figure 4).

The results showed that there were increases in the active population in all formulations (in one ml of logarithmic liquid formulation) from three months (first trimester) to six months (second trimester) after production (Table 5). In the third trimester, however, this level decreased in all formulations, with no significant changes in the fourth compared to the third trimesters (Table 5). In the fifth trimester of production, active population (spore growth) was observed in LF-8 only (Table 5).

Based on the results during the investigated period, active population peaked in the second trimester with the highest active population belonging to liquid formulations of LF-1 (CDB + 30 °C + NaNO3), LF-2 (CDB + 25 °C + $NaNO_3$), LF-3 (CDB + 30 °C + D-cycloserine), and LF-8 (PDB + 25 °C + D-cycloserine) (Table 5).

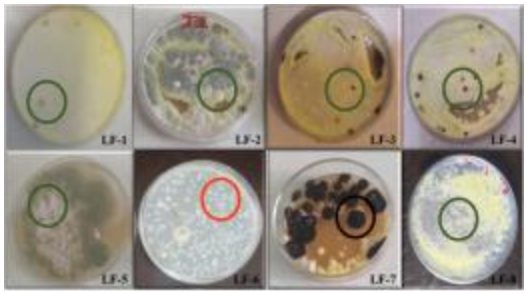

Figure 4. Active population of *T. flavus* in liquid formulations (LF-1, LF-2, LF-3, LF-4, LF-5, LF-6, LF-7, and LF-8) in the second trimester post-production. Colony of *T. flavus* (green circle), and infections with *Aspergillus niger* (orange circle) and *Penicillium* spp. (black circle)

Table 5. Comparison of *T. flavus*active population in 1.0 ml of liquid formulations at 4 °C

T. flavus liquid formulations	First trimester		Second trimester		Third trimester		Fourth trimester		Fifth trimester	
	Logarithmic spore count	Spore count/ml (× 10^9)	Logarithmic spore count	Spore count/ml (× 10^9)	Logarithmic spore count	Spore count/ml (× 10^9)	Logarithmic spore count	Spore count/ml (× 10^9)	Logarithmic spore count	Spore count/ml (× 10^9)
LF-1 (CDB + 30 °C + NaNO3)	6·574e	0·0037	7·210b	0·0160	6·138b	0·0018	6·138b	0·0018	—	0

LF-2 (CDB + 25 °C + NaNO3)	6·653e	0·0045	7·342b	0·0220	6·096b	0·0012	6·096c	0·0012	—	0
LF-3 (CDB + 30 °C + D-cycloserine)	6·875d	0·0075	7·574a	0·0275	6·096b	0·0012	6·096c	0·0012	—	0
LF-4 (CDB + 25 °C + D-cycloserine)	5·698f	0·0005	6·096c	0·0012	5·301e	0·0002	5·301e	0·0002	—	0
LF-5 (PDA + NaNO3 + 30 ºC)	8·204a	0·1600	5·574d	0·0003	5·759c	·000570	5·740d	·000550	—	0
LF-6 (PDA + NaNO3 + 25 ºC)	7·380c	0·0240	5·602d	0·0004	5·698cd	·000500	5·698d	·000500	—	0
LF-7 (PDA + D-cycloserine + 30 ºC)	7·698b	0·0500	5·243e	0·0001	5·602d	0·0004	5·602d	0·0004	—	0
LF-8 (PDA + D-cycloserine + 25 ºC)	6·774d	0·0059	7·096b	0·0120	6·720a	0·0052	6·698a	0·0050	5·880	·000760

* LF: Liquid Formulation, ** CDB: Czapeck Dextrose Broth, *** PDA: Potato Dextrose Broth

• There is no statistically significant difference between means with similar letters at 1% level.

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Determining the contamination rate of T. flavus liquid formulations within15 months post production at 25 °C

Bacterial and fungal (*A.niger* and *Penicillium* spp.) infections were observed in different formulations following 15 months of production at 25 °C (Figure 5). The highest levels of contamination were calculated for LF-5, LF-6, and LF-7 formulations from the first to the third trimester, during which the contamination percentage in different formulations ranged from 0.67 to 70% (Table 6), and reached 70% and 80% for all formulations in the fourth and fifth trimesters, respectively (Table 6).

This experiment was significant at 1% level according to the statistical analysis of data (ANOVA) for the first to third trimesters after production. Comparison of means revealed the lowest contaminations in the mentioned period belonged to formulations containing CDB medium (LF-1, LF-2, LF-3, and LF-4) and LF-8 (PDB + D-cycloserine + 25 °C (Table 6).

Figure 5. Bacterial and fungal contaminations of liquid formulations in the fifth trimester at 25 °C. Infection with *Penicillium* spp. in LF-7, bacterial infection in LF-6, and bacterial and fungal (*A. niger*) infections in LF-5.

Table 6. Comparison of mean infection rates of different *T. flavus* liquid formulations at 25 °C

T. flavus liquid formulations (LF*)	First trimester infection (%)	Second trimester infection (%)	Third trimester infection (%)	Fourth trimester infection (%)	Fifth trimester infection (%)
LF-1 (CDB + 30 °C + NaNO3)	1·33c	3·59b	4·25d	70	80
LF-2 (CDB + 25 °C + NaNO3)	1·33c	5·40b	5·15d	70	80
LF-3 (CDB + 30 °C +	1·00c	3·59b	6·25d	70	80

D-cycloserine)					
LF-4 (CDB + 25 °C + D-cycloserine)	1·00c	1·33b	20·51c	70	80
LF-5 (PDA + NaNO3 + 30 ºC)	2·92b	20·51a	70·00a	70	80
LF-6 (PDA + NaNO3 + 25 ºC)	2·88b	2·88b	71·66a	70	80
LF-7 (PDA + D-cycloserine + 30 ºC)	4·75a	3·45b	60·00b	70	80
LF-8 (PDA + D-cycloserine + 25 ºC)	0·89c	3·18b	3·18b	70	80

* LF: Liquid Formulation, ** CDB: Czapeck Dextrose Broth, *** PDA: Potato Dextrose Broth

• There is no statistically significant difference between means with similar letters at 1% probability level.

Determining the contamination rate of T. flavusliquid formulations within 15 months post-production at 4 °C

Bacterial and fungal (*A.niger* and *Penicillium* spp.) infections were observed from the second trimester. In the second and third trimesters, these infections were 20 and 30%, only in LF-5, LF-6, and LF-7,respectively. The infections in all formulations accounted for 40% and 60% in the fourth and fifth trimesters, respectively. At this stage, therefore, no statistical analysis was required for the results.

(Figure 6)shows the bacterial and fungal (*A.niger* and *Penicillium* spp.) infections in LF-5, LF-6, and LF-7 formulations in the fifth trimester.

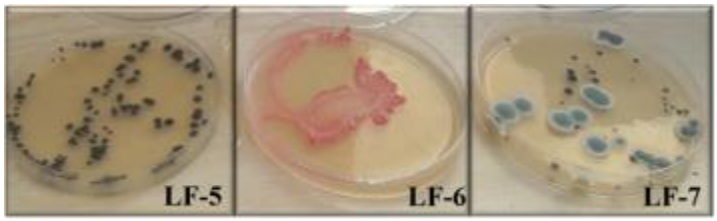

Figure 6. Bacterial and fungal infections of liquid formulations in the fifth trimester at 4 °C; Infection with *Penicillium* spp. in LF-7, bacterial infection in LF-6, and bacterial and fungal (*A. niger*) infections in LF-5.

DISCUSSION

The overall results of this study demonstrated that the four formulations of LF-1 (CDB + 30 °C + NaNO3), LF-2 (CDB + 25 °C + NaNO3), LF-3 (CDB + 30 °C + D-cycloserine), and LF-8 (PDB + 25 °C + D-cycloserine) were the most effective liquid formulations in terms of maximum mycelial mass, sporulation, *T. flavus* active population, and lowest infection rate.

In sporulation study, it was found that spore counts increased in some formulations at4 ºC up to the second trimester afterproduction. According to available literature (Cooney and Emerson, 1964), a temperature range of4-5 ºC to 55 ºC was reported for growth and proliferation of this fungus despite the thermophilicity*T. flavus*(Scott and Bernard, 1987).

Examinations of mycelial mass, sporulation, and active population indicated that the formulations containing CDB were generally more successful, which can be attributed to two reasons. The first is that among the factors (culture medium, temperature, and stabilizers) used in the formulation, the individual factors of PDB medium (Marois et al., 1984), 30 °C (Marois et al. al., 1984), and NaNO3 stabilizer were more efficient inthe growth and stability of *T. flavus* compared with CDB medium at 25 °C and dicycloserin estabilizeras demonstrated in previous research. The second reason is that the accumulation of factors affecting the growth and proliferation of fungi results in the "crowding effect" phenomenon followed by the growth inhibition by the fungus itself (Chitara et al., 2004).

Accordingly, it can be deduced that when weak and strong factors of a formulation conjoined in the fungal proliferation and growth, that formulation was more effective in fungal growth and proliferation in our research. This study also revealed that the formulations containing the weak factor of CDB medium (LF-1, LF-2, and LF-3) couldcooperate with the other two factors (temperature and stabilizers) in the proliferation and growth. LF-8 formulation with the strong PDB medium together with two weak factors (25 °C and D-cycloserine stabilizer)could influence the increased sporulation, stability, and mycelial mass weight. However, reduced sporulation rate and active population were observed in formulations with strong PDB medium together with the other two strong agents (LF-5 with 30 °C and NaNO3 stabilizer) and even other strong factors (LF-6 with 25 °C and NaNO3 stabilizer; LF-7 with 30 °C and D-cycloherine stabilizer).

LF-4 formulation also experienced decreased sporulation and active population of *T. flavus* with the presence of three weak factors (CDB medium, 25 °C, and D-cycloserine stabilizer). Our observations indicate that, regardless of the time as a factor affecting the contamination of various media including liquid ones, the lowest contamination occurred in the formulations (LF-1, LF-2, LF-3 and LF-8) with higher levels of sporulation and active populations up to nine months (third trimester) after production due to the principle of fungal competition. This principle suggests that bacterial and fungal saprophytic infections occur at times of declined fungal population (Shearer, 1995).

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