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Original Article

Isolation, Identification and Growth's Comparison f Mold Types In A Cake Factory Environment And Final Products

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ABSTRACT

Objective: Mold contamination of cakes and the possibility of mold growth can pose a serious health problem. Bakery products include various ingredients, which regularly contain molds. These microorganisms on cakes can come from the air, contaminated packaging materials and other sources. **Methods:** In this study, a total of 500 fungal isolates representing 36 species, were identified from cake samples and ambient environment air on four different media. For each cake sample, four culture and two culturing methods, spread plating and pour plating, were applied. For environment, precipitating technique during producing and suspension of work were used. Identification based on morphological characterization included colony's structure and profiles were identified by slide culture technique and primary Keys identification. Meanwhile four type's media studied for growth rate (mm/day) of 36 isolated mold species after 14 days at 25°C. **Results:** The most predominant fungal genera arranged in cake samples were Aspergillus sp., Penicillium sp., Mucor and Rhizopus sp.as the same, and Alternaria sp. respectively. The most frequent environment fungi were as the order follows: Aspergillus (24.5%), Penicillium (21.30%), Cladosporium (20.65%), Alternaria (12.15%), Trichoderma (9.51%), Epicoccum (7.29%), Mucor (3.64%), Rhizopus (0.81%). No significant difference was observed between the data obtained the spread plating and pour plating technique. Spores germination for each media was also measured and indicated varies greatly between MEA and other. **Conclusions:** Comparison of colony's growth rates was adapted with the previous results of counting colonies. Considering the time-rate relationship amongst 36 fungal species, 14 of them fallowed linear equations and 7 others followed nonlinear Gauss-Newton curves.

1.INTRODUCTION

Bakery products includes various ingredients such as cereal flour, cocoa powder, sugar, egg, ,edible oil, nuts, dried fruit, spices which regularly contain molds (Levic et al., 2004; Samson et al., 2004; Pitt and Hocking, 2009; Kocic-Tanackov and Dimic, 2012). Furthermore, the fine bakery product comprises an intermediate or high moisture baked product having a water activity (aw >0.8), which is sufficient to keep the product mold free when packaged for a storage time of 2 weeks or more at ambient temperature (Samet and Spengler, 2003). Baked products with a relatively neutral pH, high moisture content and water activity such as cakes, muffins, waffles, and tortillas are particularly prone to rapid spoilage from a variety of molds, principally Penicillium and Aspergillus species (Cook and Johnson, 2009). These microorganisms on cakes can come from the air, contaminated packaging materials and

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other sources. The bioaerosols (airborne fungi and bacteriain) food production environments air is an important potential source of microorganisms, including pathogens (Wray, 2011). Airborne contamination of product is a combination of the microbial load of the air, the time the product is exposed to the air (sedimentation from the air in still conditions) or as an interaction with the air if the air is used for, cooling or fluming of the product (Brown and Wray, 2014).

Many industrially produced baked goods emerge from the baking process with a surface that is essentially sterile but post bake handling can quickly lead to fungal surface contamination as a result of exposure to airborne contaminants as well as equipment contact. Following surface contamination, many baked goods are then very vulnerable to surface mold spoilage, the severity of which is linked to factors such as the degree of contamination, the moisture content of the product and the storage conditions (Graham, et al. 2006).

Mold contamination of cakes and the possibility of mold growth can pose a serious health problem. The fact that molds produce a large amount of spores that are easily disseminated provides explanation of the wide distribution in nature and herbal products. The role of mold spores and mycelium as a source of allergenic diseases has been widely described (Gutarowska and Zakowska, 2002). It has been estimated, that 5–30% of atopic symptoms may be the result of prolonged exposure to allergenic spores in air. The main molds present in indoor air are those found growing in the building materials, Cladosporium, Alternaria, Aspergillus, Penicillium, Fusarium, Ulocladium, Aureobasidium, Cephalosporium, Trichoderma, Sistotrema to be cause of asthma, extrinsic allergic alveolitis, and allergic dermatitis (Flannigan et al., 1991; Godish et al., 1996; Miller and Joung, 1997). Several thousand mold species are strong allergens. Respiratory allergy is widespread among all age groups but its main effects are on children (Diaz, 2005; Kocic-Tanackov and Dimic, 2012). Toxigenic molds as Aspergillus sp., Penicillium, Penicillium, sp. may also be present in indoor air and on the surface of building materials (Flannigan et al., 1991; Johanning et al. 1996; Nielsen, 1999).

According to the available literatures, there are very few data on mold contamination of different types of cakes. Therefore, the objective of this study was to survey contamination of mold processing plant environment, its products, determine and identification the presence of the main fungal species. Besides, to devise a mathematical model, which could describe a correlation between grow in grate of fungal species isolated with time. It has been proved that the elaborated model is useful for the evaluation of growth rate of mold by comparing size of colonies on four cultures with the time.

2. MATERIALS AND METHODS

2.1. Samples of cakes and environment

Cake samples were randomly collected on factory located in the industrial town of Mashhad (Khorasan razavi, Iran). 12 samples (named A to L). Also, flour used in the cake factory as one of the main building materials, was selected as sample No. 13 (namely M).

First, a pilot study of the sampling, 8 sampling locations were selected for evaluation. Air sampling was carried out in different parts of the plant: cooling, cooking, warehouse, packaging, weighing and laboratory. Carton box packaging two steps of air sampling were performed: Air was sampled before the beginning and during production. Air sampling (representing the actual air contamination) for fungi can be done by three standard methods including: impactor, liquid impinger, and air filtration methods. Gravitational settling is a much earlier approach to collect the particles that settle passively on the open petridish containing the growth medium (Bartlett et al., 2004; Cook et al., 2011). For sampling, precipitating method (fall spores in petridish), that is the way of separation airborne fungi were used.

2.2. Isolation and determination of the total number of molds in cake

For each sample, 2 culturing methods (spread plate and pour plate) were applied. This involved two procedures: Preliminary, under aseptic conditions, each sample was homogenized, ten g of each cakes were weighed and homogenized in 90 mL of 0.1% sterile peptone water (0.1 g of peptone/100 mL of distilled water). The next, in the spread method, after 30 min of orbital shaking (100 rpm) (Unimax 1010, Heidolph, Germany), o.1 ml of each suspension and dilutions (10^{-1} and 10^{-2}) were spread on 8 cm Petri dishes. Each trial involved two replicate. The other method 1 ml of each dilution in duplicate was deposited in a Petri dish (80 mm diameter), and the culture medium was poured over it following the dilution plates method. Plates were incubated in an upright position at 25°C. After 3, 5 and 7 days incubation, plates on which l0-100 colonies had

formed were selected for separately enumerating yeasts and molds. Total number of molds was determined by the dilution method. The colonies were counted and expressed as CFU/g (Pitt and Hocking, 1997). Tests were repeated in four culture media: potato dextrose agar (PDA), malt extract agar (MEA), dichloran (18%) glycerol agar (DG18) and Yeast Extract Glucose Chloramphenicol Agar (YGC) (Braendlin, 1996).

2.3. Isolation and determination of the total number of molds in cake factory environment

In this study, initially plates placed in determined location; Then for 20 minute opened, thereupon the lid plates was closed with parafilm and incubated at 25 °C for 7 to 10 days. Same colonies were similar; the same issue was considered and identified to the genius level, based on the colony morphology (Bartlett et al., 2004; Cook et al., 2011).

2.4. Mold identification

After determining the total number, in addition to morphological structure, mold and yeast colonies were identified by slide culture. Sub-culturing of isolated colonies on PDA, DGl8, MEA and YGC; followed by macro and microscopic identification. Determination of each species of fungi and mycological analyses were performed with using the keys and previously described (Klich and Pitt 1988; Pitt and Hocking, 1997). The type of sporulation and the morphology of the spores and spore-bearing structures are keys characteristics in fungus identification (Heath, 1995). This was done by observing both macroscopic characteristics of the colonies on various media used as well as the microscopic morphology and measurements of the conidiophores (after staining mycelia with 0.1% fuchsine dissolved in lactic acid) under an Olympus B061 Compound microscope (Wirsam Scientific, S. Africa) and microscope equipped with a Dino Camera Ser. No. 208060245 and Dino capture software (Zeiss, West Germany). Frequency and share of certain genera or species of molds in food samples were calculated according to the following equations (Kocic-Tanackov and Dimic, 2012) :

$$\text{Share (\%)} = \frac{\text{number of isolates of a genus or species}}{\text{total number of isolates of all genera or species} * 100}$$

$$\text{Frequency (\%)} = \frac{\text{number of samples where the genus were identified}}{\text{total number of samples} * 100}$$

2.5. Growth rates

36 fungal species isolated from cake sample and air sampling sites collaborated in this study. The radial growth rate was determined by periodical measurement of two right-angled diameters of the colonies. Radial growth rate vs. time was plotted and were evaluated from the slope by linear regression on four media PDA, MEA, DG18 and YGC (Braendlin, 1996; Patriarca et al., 2011). All the experiments were performed in duplication. An average radial growth rate was calculated from the different independent experiment.

2.6. Statistical analysis

All statistical analyses were performed using MINITAB (version 16.2.0; LEAD Technologies, Inc.). Analysis includes ANOVA for media, species and time also Correlation and Regression for growth rate and Tukey mean analysis.

3. RESULTS AND DISCUSSION

3.1. Identification of fungal contamination in cake sample

Based on the results obtained from four different media, mold's total count range for was $3.4\text{-}6 \times 10^5$ cfu/g. Only there was no growth in samples no 5, 9, 10, 11 and 12. In addition, no significant difference was observed between the data obtained the spread plating and pour plating technique ($p<0.05$). Five contaminating fungal genera were identified: Aspergillus, Penicillium, Mucor, Rhizopus, Alternari and yeast. Table 1 indicates that Aspergillus and Penicillium genera were found in all of the samples with the highest frequency for Aspergillus spp. followed by Penicillium spp. Mucor spp. and Rhizopus Genera, were only found in flour at a frequency lower than 10^3cfu g^{-1}. Mucor spp. only on DG18 media and Rhizopus Genera were observed on YGC.

Table 1:

Frequencies (%) of mold genera in cake samples

Genus	Number of tested samples /number of contaminated samples	Share (%)	Genus frequency (%)
Aspergillus spp.	13/8	98.25	61.54
Penicillium spp.	13/8	1.41	61.54
Rhizopus	13/1	0.04	7.96
Mucore spp.	13/1	0.24	7.96
Alternaria spp.	13/1	0.02	7.96
Cladosporiumspp	ND	ND	ND
Tricoderma spp.	ND	ND	ND
Epicocume spp.	ND	ND	ND

ND: not detected

According to morphological characters, the most of fungal species of cake samples were belonging to Aspergillus spp., included: *A.candidus* (0.8%), *A.glacuse* (19.8%), *A.nidulance* (3.5%), *A.parasiticuse* (38.5%), *A.penicillioides* (12%), *A.tereus* (1.2%), *A.flavuse* (7.3%), *A.fumigatuse* (6%), *A.ocraseuse* (9.8%) and *A.vesicolore* (0.9%). This is first report on identification of large number of Aspergillus strains isolated from cake sample in Iran. Figure 1 display picture macroscopical and microscopical characters of two main Aspergillus species on four media.
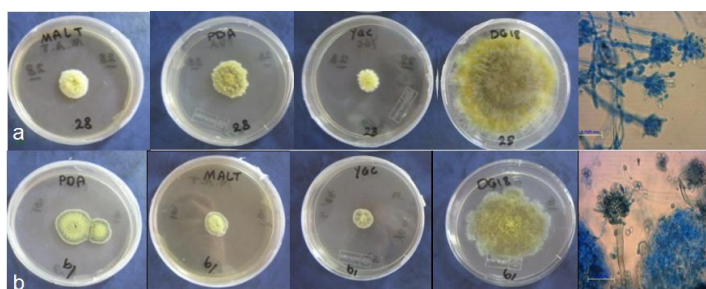

Figure 1: picture of macroscopical and microscopical characters of two main Aspergillus species on four media (DG18, PDA, YGC and MEA): (*a):A.parasiticuse*, (b): *A.glacuse*

The taxonomical identification of mold was based on their morphological characteristic as observed under compound microscope after 1-2 week at 25°C. Morphological characters were studied for identification of all these isolates along standard cultures four differential culture media. In addition, Based on the results of morphological characters of Penicillium spp. were subtended: *P.chrysogenum*, *P.viridicatum*, *P.commune* and *P.digitatum* (Fig2)*.
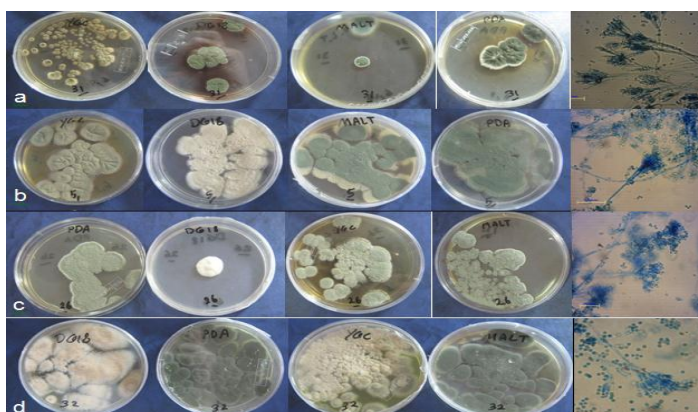

Figure 2: pictures of macroscopical and microscopical characters of Penicillium species on four medias the left to right (DG18, PDA, YGC and MEA): (a):*P.chrysogenum*, (b):*P.viridicatum*, (c): *P.commune*,(d): *P.digitatum.*

The presence of *A.glacuse*, *A.parasiticuse*, *A.penicillioides*, *A.tereu*, *A.flavuse*, *A.fumigatuse, A.ocraseuse, R. stolonifer, mucor* spp. and *P. citrinum* on these products might be due to spores are to some extent more resistant to dry conditions than the vegetative mycelia (Smith, 1960). The presence of these molds on cake sample may result in production of toxic substances which could lead to health hazard for the consumers (Aletor, 1990).

Similar to the findings reported by Abdel Hameed, (2007) Cladosporium, Aspergillus and Penicillium were considered to be allergenic fungi frequently found in flour mills. These findings were also supported by Kocic-Tanackov and Dimic, (2012) study, where Aspergillus improper handling during processing. Some of these fungi, especially Aspergillus spp. are able to survive in situations where free water is not available (Samson et al., 1992), especially their (29.75%) and Penicillium (51.25 %) were the major fungal types found in the cake sample, while genera Fusarium, Alternaria, Cladosporium and Eurotium accounted for 7.75%, 4.50%, 2.75% and 1.50% of the total population of cakes, Other genera (Acremonium, Mucor, Phialophora, and Ulocladium) were represented per one species and accounted to a smaller percentage (2.5%) of the isolated fungi. In the following figure are two isolated Rhizopus and mucor macroscopic and microscopic images are shown (Figure 3).
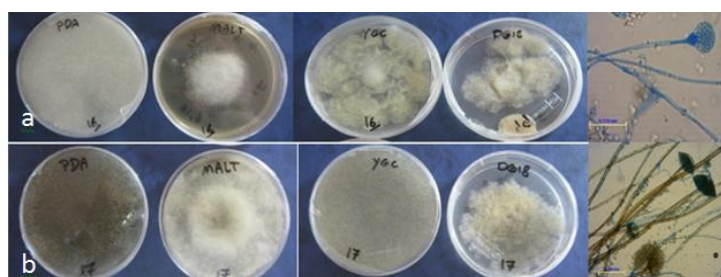

Figure 3: pictures of macroscopical and microscopical characters of Mucor (a) and Rhizopus (b) species on four medias the left to right (PDA, MEA , YGC and DG18)

By comparing the data obtained for the number of molds on certain media, it can be concluded that the overall number was dependent on the type of the medium used for their isolation, and the presence of fungal contamination. Given that the most frequent species were from the groups of moderately xerotolerant molds (Penicillium, Aspergillus).

The mold total count reported in Figure4. Based on ANOVA and Tukey analysis test, there was significant different among media that labeled in Fig4. As a result DG18 is the best media for identification of mold contamination that in Iranian standard's for baked product advised using this media.
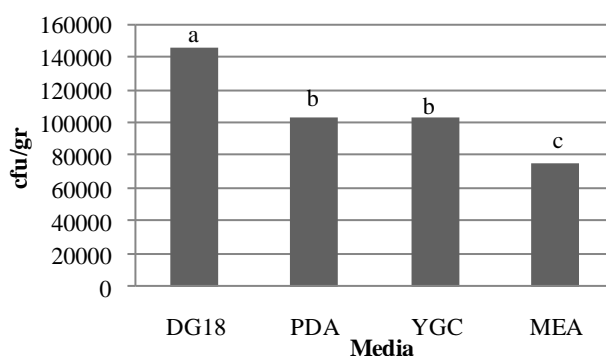

Figure 4: The average number of molds (cfu/gr) on DG18, YGC, MEA and PDA media

There is a linear dependence with high values of correlation coefficients between the total number of molds on DG18, YGC, MEA and PDA medium (Figure 5). This indicates that the determination of the total number of molds on one medium can with great reliability predict the total number of molds on the other tree media for the tested samples of cakes. There was six equation for predict regression in various media with R-sq range between 0.86 to 0.99.
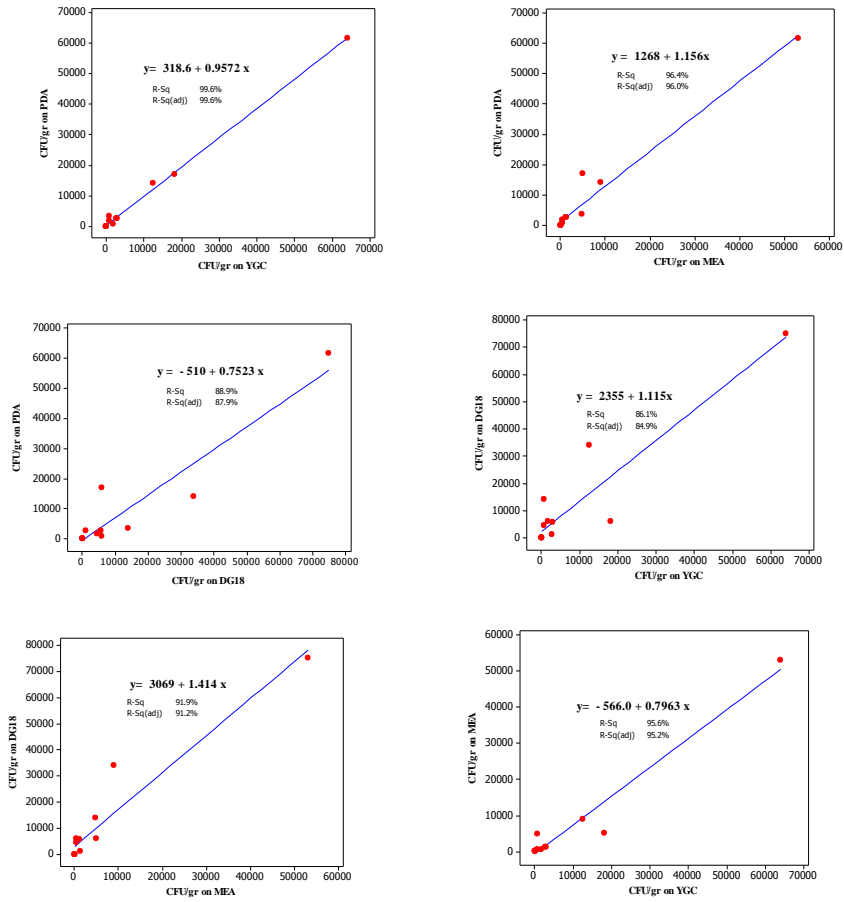
Figure 5: Linear regression equations of total number of molds on DG18, YGC, MEA and PDA media

3.2. Identification of fungal contamination in air sampling sites

Penicillium spp. (*P.viridicatum*, *P.italicum*, *P.citrinum*, *P.digitattum* and *P.verococosum*), Aspergillus spp. (*A.utuse*, *A.carbonarius*, *A.flavuse*, *A.tereuse* and *A.versicolore*), *Cladosporium cladosporioides* and *Alternariain fectoria*, were dominant fungi species found in the indoor and outdoor factory environment, with relative frequencies of 24.5%, 21.30%, 20.65% and 12.15%., respectively. Other species of fungi were included, *Trichoderma harzianum* (9.51%), *Epicoccum higrum* (7.29%), Mucor spp. (3.64%), *Rhizopus astolonifer* (0.81%) and yeast (0.75%). Figure 6 shows macroscopical and microscopical characters of species that have been only isolated from environment that not exist in cake samples.
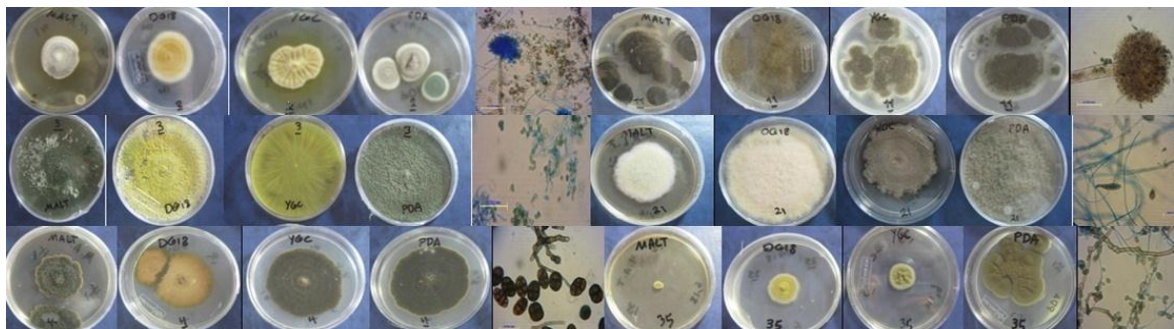

Figure 6: macroscopical and microscopical characters of fungal species order from left to right: *A.utuse, A.carbonarius, Trichoderma harzianum, Alternaria infectoria, Epicoccum higrum, Cladosporium cladosporioides*

Meanwhile, more than 60% of total fungal variety in environment consisted of fungal variety in cake sample (Table 2). In this study, the highest fungal contamination in cake sample were Aspergillus spp., while the highest levels of fungal contamination in the environment is related to penicillium spp. As a result, although Penicillium spp. and Aspergillus spp. exist in air as the same number, but in cake samples Aspergillus spp. dominated that shows Aspergillus spp. can germinate and growth better than penicillium spp. in cake samples. Probably Aspergillus spp. can tolerate and more growth in dry places related to penicillium spp.

Table 2:

Occurrence of fungal species in cake samples and environment product

Fungal strains	species	source		Fungal strains	species	source	
		Envi	cake			Envi	cake
Aspergillus sp.	*A.candidus*	-	+	Aspergillus sp.	*A.penicilliodes*	+	+
	A.utuse	+	-		*A.ocraseus*	+	+
	A.glacuse	-	+	Penicillium sp.	*P.viridicatum*	-	+
	A.nidulance	-	+		*P.viridicatum*	-	+
	A.glacuse	-	+		*P.italicume*	+	-
	A.carbonariuse	+	-		*P.citrinum*	+	-
	A.parasiticus	-	+		*P.commune*	-	+
	A.nidulance	-	+		*P.digitatum*	+	+
	A.penicilliodes	+	+		*P.chrysogenume*	+	+
	A.parasiticus	-	+		*P.digitatum*	+	+
	A.tereus	+	+		*P.verocosum*	+	-
	A.flavuse	+	+		*P.italicume*	+	-
	A.flavuse			Mucore sp.	-	+	+
	A.fumigatuse	-	+	Rhizopus sp.	*R.astolonifer*	+	+
	A.parasiticus	_	+	Cladosporium sp.	*C.cladosporioides*	+	-
	A.tereus	+	+	Tricoderma sp.	*T.harizianum*	+	-
	A.ocraseus	+	+	Epicoccum sp.	*E.higrum*	+	-
	A.versicolore	+	+	Alternaria .sp.	*A.infectoria*	+	+

There are many reports on fungi isolated from indoor environments, for instance: According to the study carried out by Tsai and Liu (2009), to evaluate the levels of microorganisms to minimize the biological hazards, Aspergillus, Penicillium, Cladosporium, were mainly fungal species isolated from noodle factory, Nantou, Taiwan. Kim et al. (2009) were investigated the distribution patterns of airborne fungi of feedstuff manufacturing factories at Seoul Korea, Aspergillus, Penicillium and Cladosporium showed mucha higher level of fungal contamination than other fungal genera species. In the bakery at Bucharest Romania, Similar observations were made by Cornea et al. (2011), they used Conventional and molecular methods to detect predominant fungal species of bakery environment, which included: Aspergillus spp. Penicillium spp. Alternaria spp. Fusarium spp.

The levels of fungi in clean areas such as the packaging site, baking and lab site were the lowest. Conversely, as for general areas, serious fungal concentrations at the warehouse site and carton packaging site exceeded the guideline of 100 cfu/m3. In this study, the frequency of common allergy-inducing molds was ordered as follows: Cladosporium, Penicillium and Aspergillus, in environments. Since airborne fungal spores could penetrate doors and windows, it was proposed that fungal flora indoors represented the outdoor atmosphere (Chew et al., 2003). These findings were also supported by Abdel Hameed (2007) study, where Aspergillus and Penicillium were the major fungal types found in the interior of a flour mill, while Cladosporium and Aspergillus where the main fungi found outside the mill.

Fungal contamination found in the air of cake manufacturing factory before and during the work activities periods. According to the t test, by comparing the fungal contamination of two steps, the mean contamination were statistically different higher in during compared to those found before work activities (Fig8). Youn kim et al. (2006) reported similar results, Also, this was consistent with the previous reports that airborne contamination of product is thus a combination of the microbial load of the air, the time the product is exposed to the air (sedimentation from the air in still conditions) or as an interaction with the air if the air is used for, cooling or fluming of the product (Brown and Wray, 2014).

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3.3. Growth rates

Growth rate of fungal species obtained from ambient air and cake samples were also compared on the four media during 14 days at 25°C (Table 2). By comparing the data obtained from ANOVA and Tukey analysis test. It can be concluded that, the maximum growth rate of colonies was noted on the DG18 medium, and the lowest on the MEA. A significant difference was not between growth rate on PDA and YGC ($p < 0.05$) (Figure 7).
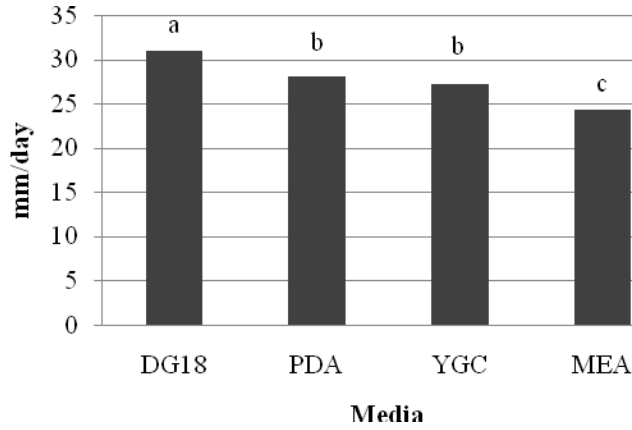

Figure 7: Comparison of growth rate of molds on DG18, YGC, MEA and PDA media

In addition, the data collected in this study by means of ANOVA test indicated significant difference between time and rate that Tukey test labeled as Figure8.
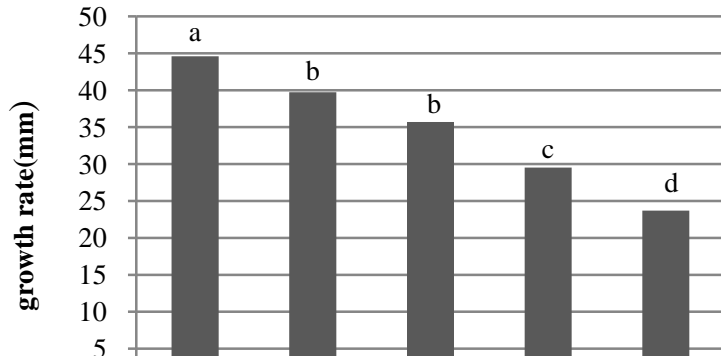

Figure 8: Comparison of growth rate of molds in during 14 days

Growth rate increased with increasing time, whereas, statistically between 10th to12th on the growth rates were not significantly different, which indicates that growth rate has been fixed in during days.
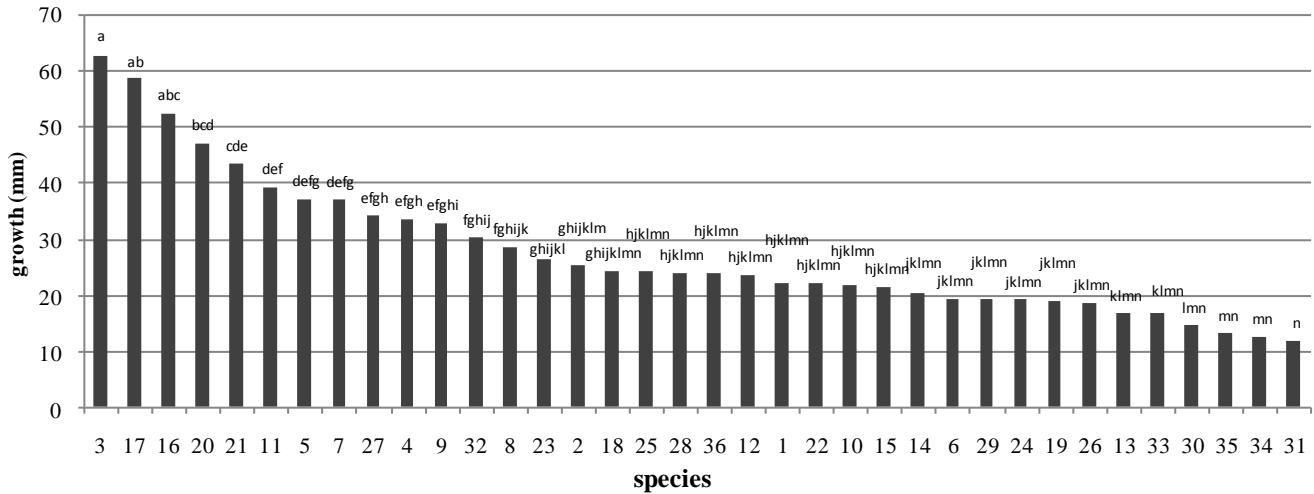
Figure 9: Comparison of growth rate of fungal species

Based on the ANOVA and Tukey's test results, there was significant difference between the growth species. The overall, three species *T.harzianum*, *R.astolonifer* and Mucor spp. had the highest growth rate than the other species respectively. According to the current measurements, in Hyaline hyphomycetes as Aspergillus spp and penicillium: growth rate of Aspergillus species were faster than penicillium spp. Between the Aspergillus species, *A.flavuse* had the highest growth rate and *A.ocraseus* the lowest rate of growth. The highest growth rates of Penicillium spp. were detected in *P.viridicatom* while the lowest growth rate was observed in *P.verocosum* (Fig9).

3.4. Evaluated equations for evolution prediction

In this study, based on the Gauss-Newton algorithm, different equation was more suitable for the growth rate curve of fungal species. Where Y is the growth rate (mm/day), X is time. The coefficient of correlation of fitting of the models function to the experimental data is $R2 = 0:87$. It should be noted that a high, statistically significant correlation between the time factor was found for some of fungal species ($r = 0:730–0:923$). The parameters of the equations for the all of fungal species isolated and each of the genera separately used in the experiments are summarized in Fig10, 11.
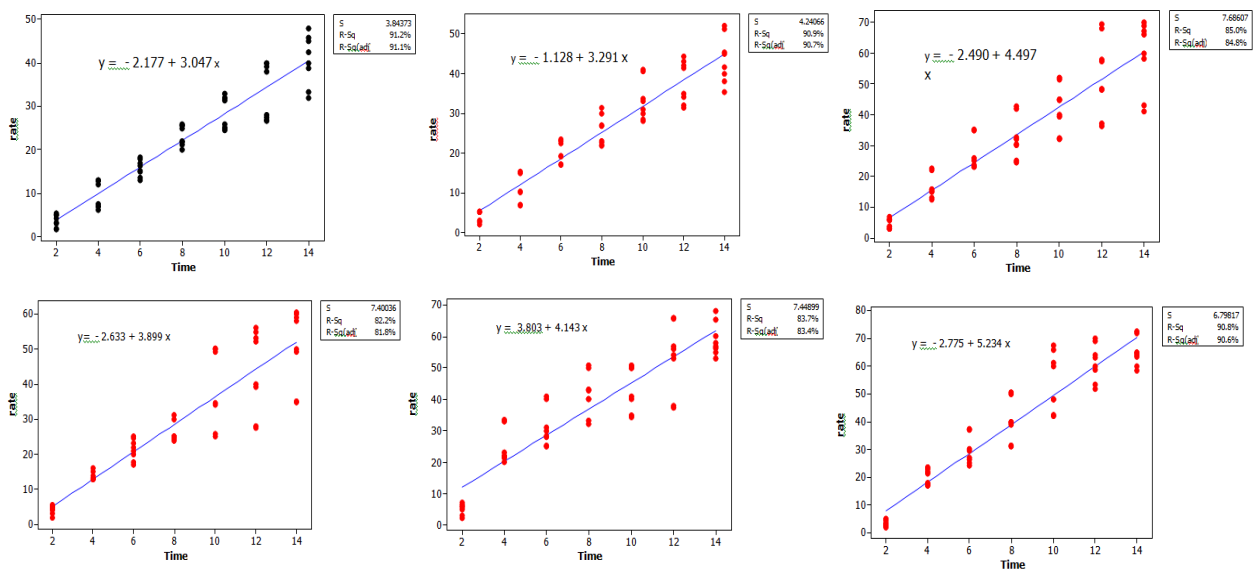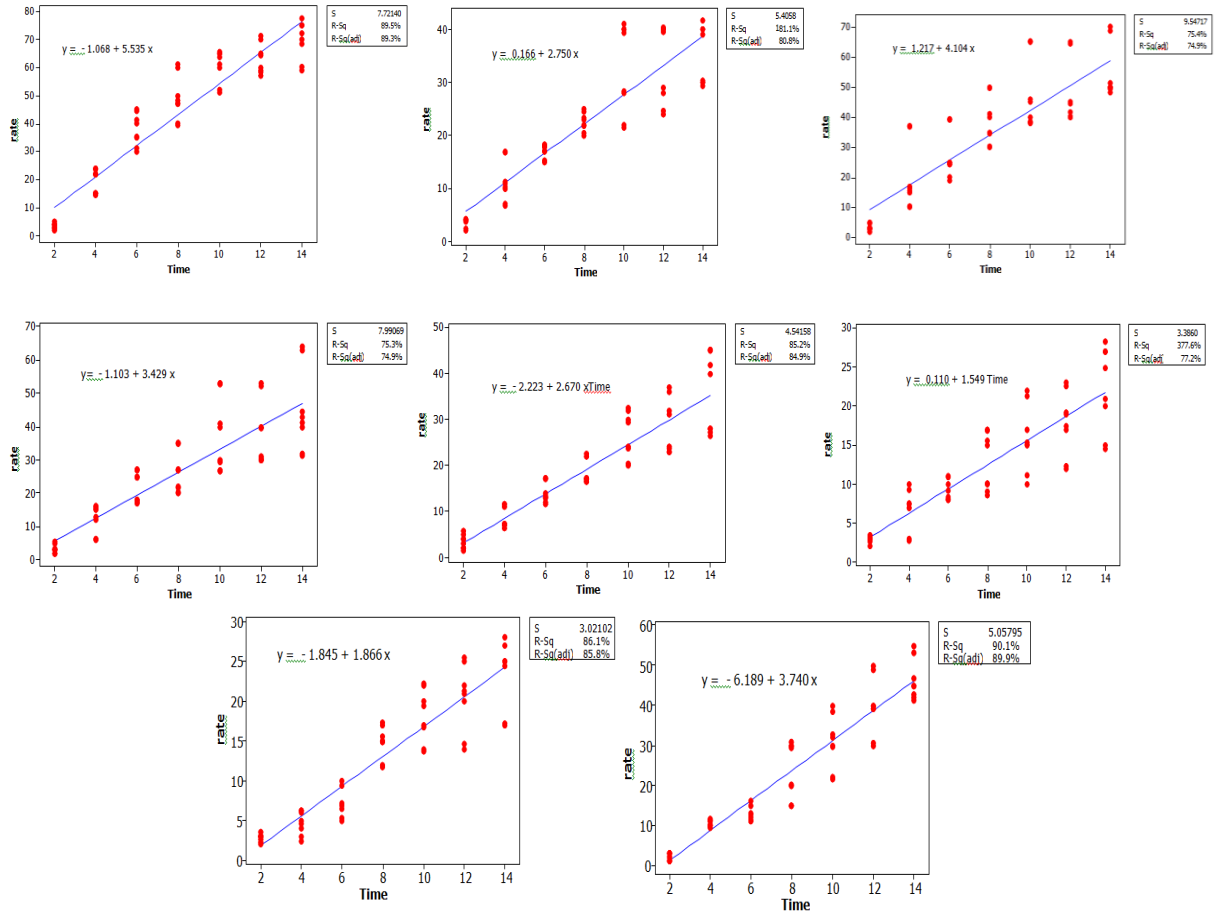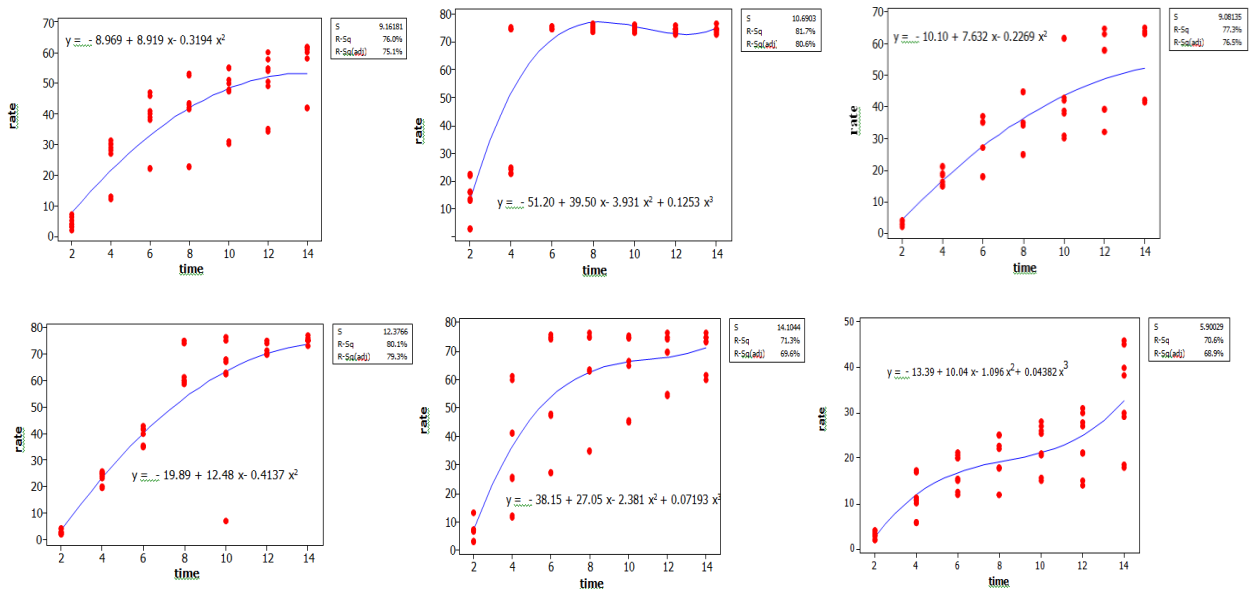
Figure 10: Parameters of the linear model equation describing correlation of time with growth rate for the fungal spp. Culturing on certain media (*correlation coefficient (r) is statistically insignificant for p<0.05)

Figure 11: Parameters of the Nonlinear model equation describing correlation of time with growth rate for the fungal spp. Culturing on certain media (*correlation coefficient (r) is statistically insignificant for $p < 0.05$)
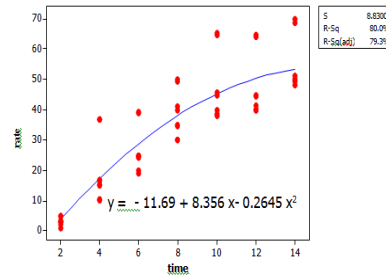
Considering the time-evolution rate relationship, amongst 36 fungal specious under experiment, 14 of them fallowed linear equations and 7 others followed nonlinear curves. Though the remaining 15 species didn't exhibit any suitable equation of either types in 4 different culture conditions under test conditions. However if the same culture

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conditions are applied, equations can also be evaluated for the 15 types mentioned. One fungal species is illustrated below as an example. Since these last 15 haven't reached any relevant equations in 4 different conditions, it can be concluded that their behavior under various culture conditions show meaningful differences
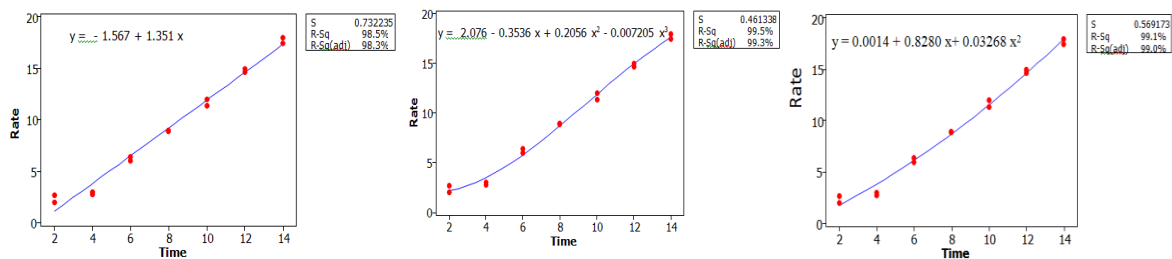

Figure 12: The results of the analyses of time and the growth rate for one media

In this paper it has been proved using statical analysis that mold elaborated on the basis of mathematical function. The presented mathematical average model describing the correlation of growth rate with time in all of

fungal species, based on this it can be used to evaluate the degree of mold activation by considering the time in a culture and can be benefit to baked goods, confectionery conmanufactuers and similar products.

desirable for germination. Bioaerosols in food production environments should be controlled using the appropriate air filters combined with production practices that minimize aerosol generation within the high-risk area. Control of airborne is important for worker health and to minimize the risk of explosions. Poor hygiene of workers, equipment and air also contributes to the total number of molds. Also, results shows equations for predicting growth rates related to time in mold types.

4. CONCLUSION

Different types of cakes that are industrial or handmade, available on the market, were contaminated with potentially toxigenic mold species. The results obtained suggest that fungal contamination and genera of toxin-producers are different between cake types and environment of factory. Additionally absence of some mold species in cake but exist in environment, shows that cake conditions isn't

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