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MONASCUS PIGMENT PRODUCTION WITH FOOD RESIDUES (STALE BREAD AND SOUR YOGHURT): A COMPARATIVE KINETIC ANALYSIS

(BAYAT EKMEK VE EKŞİ YOĞURT GİBİ GIDA ATIKLARINDAN MONASCUS PİGMENTİ ÜRETİMİ: KIYASLAMALI KİNETİK ANALİZLERİ)

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ABSTRACT

Colorants are one of the most important additives in food industry. Preference of natural colorants has become highly concerned compounds since many studies have indicated the harmful effect of synthetic colorants. The aim of this study was to investigate and compare the pigment production yield of Monascus purpureus from different food residues. Growth parameters of M.purpureus were followed during 14 days for the responses of yield, pigment profile and Monascus metabolites. Results indicated that dairy residues and stale bread could be economic, efficient and easily available substrate for M.purpureus pigment production.

Keywords: Monascus pigments, Food residues, Kinetic analysis, Natural food colorants

ÖZ

Gıda endüstrisinin en önemli katkı bileşenlerinden bir tanesi renklendiricilerdir. Sentetik renklendiricilerin zararlı etkileri ile gıdalarda doğal olan renklendiriciler tercih edilmektedir. Bu çalışmanın amacı, Monascus purpures'un ürünü olan pigmentinin çeşitli gıda atıklarının kullanılarak üretiminin incelenmesi ve kıyaslanmasıdır. Verim, pigment profili ve Monascus metabolitleri açısından M.purpureus' un üreme parametreleri tanımlı ve kompleks ortamlarda 14 gün boyunca izlenmiştir. Elde edilen bulgular, gıda atıklarından süt ürünlerinin ve bayat ekmeklerin M.purpureus ile pigment üretimi için ekonomik bir substrat olabileceğini göstermiştir.

Anahtar Kelimeler: *Monascus pigmenti, Gıda atıkları, Kinetik analiz, Doğal gıda renklendiricileri*

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1. INTRODUCTION

Many natural and synthetic colorants are used in food, textile, pharmaceutical and cosmetic industry. Since many studies indicated the serious health risks of synthetic colorants, replacing the synthetic colorants with natural colorants gained high concern and importance. As parallel to this, consumers are much more conscious and sensitive, and thus showing resistance to use synthetic ones because of their possible health threatening effects, like allergy, hyperactive behaviors and poor attention in a group of children [1-2]. The potential danger of some synthetic colorants such as indigocarmine, tartrazine, sunset yellow and allura red have been documented [3-6]. These results have stimulated the replacement of synthetic colorants by natural ones.

In recent years, there is a growing interest to obtain the biologically active compounds from natural sources. Pigments can be derived from plants, animals and microorganisms. Among them *Monascus purpureus* has been standing out due to its valuable bio-components and pigment production ability. *Monascus* is a filamentous fungus belonging to the genus *Monascus,* family monascaceae and class ascomyceta. It has the power to synthesising secondary metabolites as the bio-pigments with connected polyketide structure. *Monascus sp* produces six primary pigments, the colours of which are yellow (ankaflavin, monascine), orange (rubropunctatin, monascorubrine) and red (rubropuntantamine, monascorubramine) [7]. The major bioactive compounds of *Monascus sp.* have not only good coloring ability but also exhibit important additional bioactivities due to their antioxidative [8], cholesterol lowering [9], antimicrobial, antiproliferative and cytotoxic properties [10-13]. Thus, the implementation of *Monascus* pigment as a coloring agent in food products provides also an additional advantage of specific activities.

The pigment production efficiency of *Monascus purpureus* depends on substrate type and some environmental factors during the fermentation process. Chemically defined media has many advantages over the complex media, in which the effect of each component can be well understood. However, it is comparatively higher cost process. Thus, alternatively, easily available and valuable food components in food residues, agro industrial byproducts and wastes can be processed as a substrate to yield economic fermentation and value added high pigment output. So far many agro industrial residues and crop such as coconut oil cake, corn/corn cob, rice/rice bran, jack fruit seed, groundnut oil cake, and wheat bran were used as substrate for *Monascus* pigment production [14-15]. *Monascus purpureus* produces different secondary metabolites with varying bioactivities by different substrates [16]. Past research on Monascus purpureus has concentrated only two broad issues; pigmented rice production and their metabolites' bioactivities. Considering the issue of apprising food residues as substrates in fermentation media to produce value added products, the literature has almost limited details of kinetic explanation of the microorganism adaptation and their metabolic activities regarding the Monascus pigments production.

Against this background, the aim of this study was to investigate and compare the pigment production yield of *Monascus sp.* on different food residues. For this purpose, YGC and LM-YGC were used as a defined media to investigate the *Monascus* metabolites. Since stale bread and sour yoghurt are easily available and large amount of food residue, they were appraised as a potential substrate of *M.purpureus*. To our knowledge, in the literature there were no studies about the evaluation of stale bread and sour yoghurt for the production of *Monascus* pigments (MPs). Comparative kinetic studies were performed for the feasibility of the process, in terms

of the growth yield, product formation and type of *Monascus* metabolites.

2. MATERIALS AND METHODS

2.1. Microorganism and Growth Medium

M. purpureus was obtained from Refik Saydam National Type culture Collection (RSSK) in Turkey. Stocks of culture in freeze-dried ampoules were activated in YM broth at 30 °C for 2 days and then transferred to Patato Dextrose Agar (PDA, oxoid) and further 7 days incubation. *M. purpureus* spores were collected with a 5 mL of FTS and diluted to obtain 10^6 spores/mL. They were seeded consecutively on to the PDA and incubated for 7 days in order to keep ready for further studies.

YGC (Sigma- Aldrich) and litmus milk fortified YGC agar (LM-YGC agar) were used as defined medium. Chloramphenicol in YGC formulation inhibits the accompanying bacterial flora, but allows the growth of *M. purpureus*. In order to determine the effect of milk products on the growth of *M. purpureus*, litmus milk was added (15 %) to the YGC formulation. 2 mL of *Monascus* spores were sprayed onto the agar surface to achieve 10^{12} spores/petri surface.

2.2. Preparation of Food Residues

Food residues (moisturized stale-bread and sour-yogurt) were used as substrates to compare the pigment yield. Stale-bread was obtained from local market, which was collected in waste bin. Bread was cut into cubes of approximately 1 cm^3 in size and autoclaved for 15 min at 121 °C. Cubes were grounded to 2mm particle size using a sterile blender. Moisture of bread was measured by infrared moisture balance (AND, MX-50) at 105 °C. Sterilized distilled water was poured onto the bread particles to achieve 60 % (w/w) humidity.

Aseptically, 15 ±0.2 grams of moisturized stale bread granules were transferred into each petri dish, and then 2 mL of *Monascus* spores were sprayed homogeneously onto the granules. Sour yoghurt was collected from a local market in Istanbul, whose expire date was passed out. pH and the amount of total solids of sour yoghurt were measured. 15 mL of sour yoghurt was poured into each petri dish and 2 mL of *Monascus* spores were inoculated onto sour yoghurt.

2.3. Determination of Biomass

The biomass during the growth kinetic studies in each substrate used was directly determined by simple treatment performed by Zhang et al., 2015 [17] with a slight modification. Whole petri dish was used to determine the mycelia grown over the surface. 3 times x 3 mL of distilled water was used to collect the mycelia subsequently. They were recovered by filtering the solution immediately through eight layers of gauze. The filter residue was washed three times with distilled water. Then the yield of biomass was determined gravimetrically after drying at 70 °C overnight to a constant weight.

2.4. Extraction of Monascus Pigments

Monascus spores were collected every day from incubated petri dishes by washing a surface with 2 mL of ethyl alcohol. MPs included solution was then collected carefully by pipetting over the petri dishes. 5 times subsequent washing was performed and samples were brought together. In each sample, the volume was added up to the 20 mL with ethyl alcohol for further analysis. Prior to the spectrophotometric measurements, samples were centrifuged at 1000 rpm for 3 minutes (Hettich Rotofix 32 A).

2.5. Measurement of Monascus Pigments

Optical density (OD) of harvested pigment solution was measured at 412nm and 500 nm with a UV-visible spectrophotometer (Perkin Elmer), representing yellow and red pigment production (expressed as OD-value per gram of dry solid material_gdsm), respectively, taking account a dilution factor. Growth kinetics and consequently pigment yield were followed during 12 day fermentation.

2.6. Determination of Ethanol during the Growth Studies

Petri surface was divided into pieces with a size of 2 cm^2. Samples were taken into the test tube including 5 mL of distilled water to investigate the ethanol content during the fermentation period. Test tubes were homogenized on a vortex and centrifuged at 3000 rpm for 3 minutes. The volume of supernatant was completed to 50 mL with distilled water. After distillation of the ethanol in the sample into the $K_2Cr_2O_7$ solution, it was analyzed by titrimetric method between $K_2Cr_2O_7$ and Fe^{2+} according to the Skoog et al., 2003 [18]. Experiments were performed in three replicates and results were presented as the mean values.

2.7. HPLC Profile of Monascus Metabolites

HPLC analyses were performed in order to determine *Monascus* metabolites of Monacolin K and Lovastatin according to the method developed by Seenivasan et al., 2015 [19] with a slight modification. Agilent 1100 series HPLC equipped with Lichrospher RP C18 column (4.6 x 250 mm, 5µm) was used in the analyses. Detection was carried out by UV detector at 238 nm for Lovastatin (Monacolin K) and anologues and/or intermediates of lovastatin synthesized during the fermentation. Samples were filtered through 0.45 µm membrane filter before injection. The mobile phase was acetonitrile: water (pH was adjusted 2.5 with H_3PO_4) mixture at a ratio of 45:55 (v/v). Flow rate was 1 mL/min. Gallic acid (Sigma) as an external HPLC standard was used in relative comparison of the *Monascus* pigments.

2.8. Modelling of Growth Kinetics

The Monod equation relates the specific growth rate, µ, and substrate concentration (S), and is given by:

$$\mu = \frac{\mu_{\max} S}{K_S + S} \tag{1}$$

In order to describe growth kinetics for both exponential and stationary phases, the logistic equation is introduced:

$$\frac{dX}{dt} = \mu X\left(1 - \frac{X}{X_m}\right) \tag{2}$$

where X is the biomass concentration and X_m is the stationary phase population size, or upper biomass concentration above which bacteria do not grow. The constant K_s is the concentration of the rate-limiting substance when the specific rate of growth is equal to one-half of its maximum [20].

MPs including Monacolin K are secondary metabolites of *Monascus*. Product formation in fermentation is described by Luedeking and Piret kinetics. The product formation rate

depends upon both the instantaneous biomass concentration X and growth rate dX/dt in a linear fashion:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \tag{3}$$

where P is the product concentration; α and β are empirical constants that possibly vary with fermentation conditions. α and β also explain the growth-associated and non-growth associated product formation, respectively.

Equations 2 and 3 were integrated as shown by Equations 4 and 5, respectively.

$$X = \frac{X_0 * e^{\mu_m t}}{1 - X_0 / X_m (1 - e^{\mu_m t})} \tag{4}$$

$$P = P + \alpha (X + X_0) + \beta \frac{X_m}{\mu_m} \ln \left\{ 1 - \frac{X_0}{X_m} \left[1 - e^{\mu_m t} \right] \right\} \tag{5}$$

3. RESULTS AND DISCUSSION

In this study, *M.purpureus* was cultivated on different substrates in order to determine the MPs production performance using different food residues. The growth rate, MPs production rate and a change in ethanol concentration during the fermentation were investigated during the study. YGC was selected as a defined media in order to eliminate the other bacterial growth but allowing the *M.purpureus* growth. An effect of litmus milk on the growth was also searched. The aim was to compare pigment production by different substrates under the same conditions of incubation time and temperature (30 °C).

3.1. Biomass

Due to the distinct chemical compositions, i.e. carbon and nitrogen source, elemental richness and even moisture content, between substrates, they provided different environment for the mycelial growth, metabolite production and substrate consumption. Hence, the kinetic parameters of *M. purpureus* were apparently different depending on the substrates used. All of the substrates used in this study served suitable nutrients for the *M. purpureus* growth (Fig. 1). *M.purpureus* formed a typical colony with its orange color, whose morphological characteristics were similar with the descriptions given by Rasheva et al., 1998 [21].

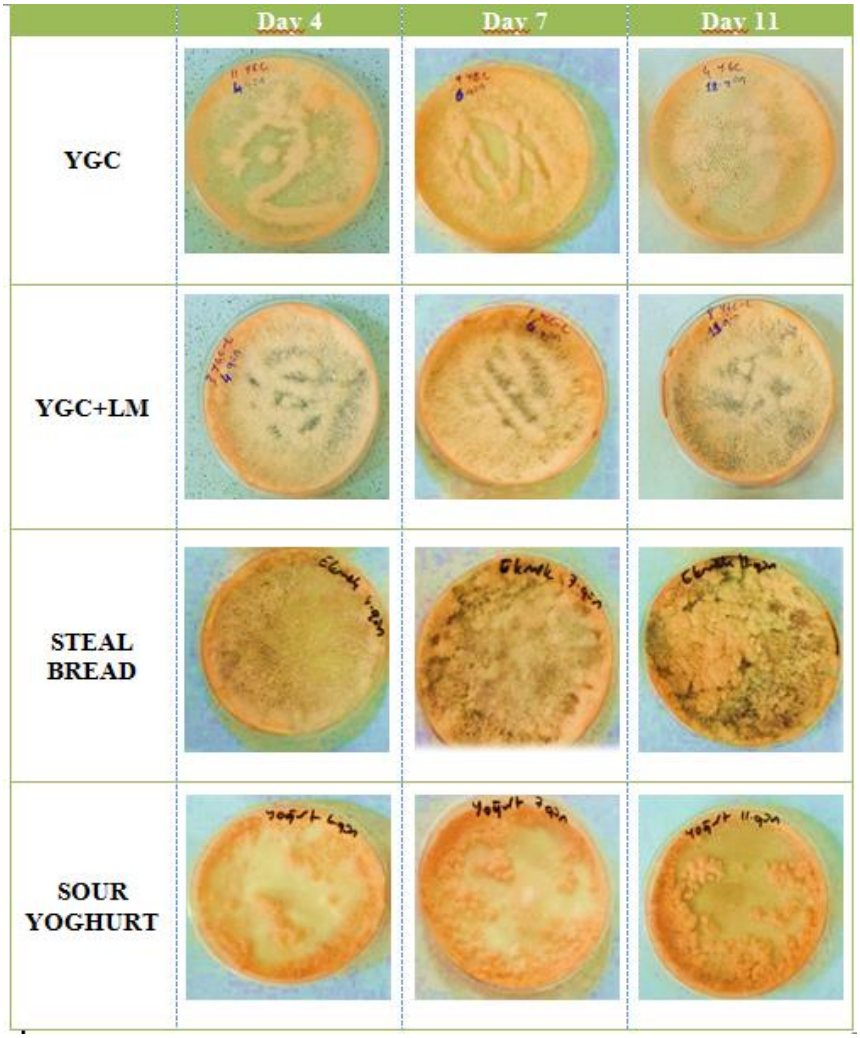

Figure 1. Growth performances of *Monascus purpureus*. on different substrates within 12 days

3.2. Growth Kinetics of M. Puppureus

In these experiment amount of inoculum was adjusted as enough to eliminate the lag phase of the growth curve. Hence, an exponential growth phase, extending from initial period to 168 h of the incubation time, and a stationary phase were seen thereafter in all of the substrates worked except stale bread (Fig.2). The biomass dry weight continued to increase to a highest value until 240h of incubation period when stale bread was used.

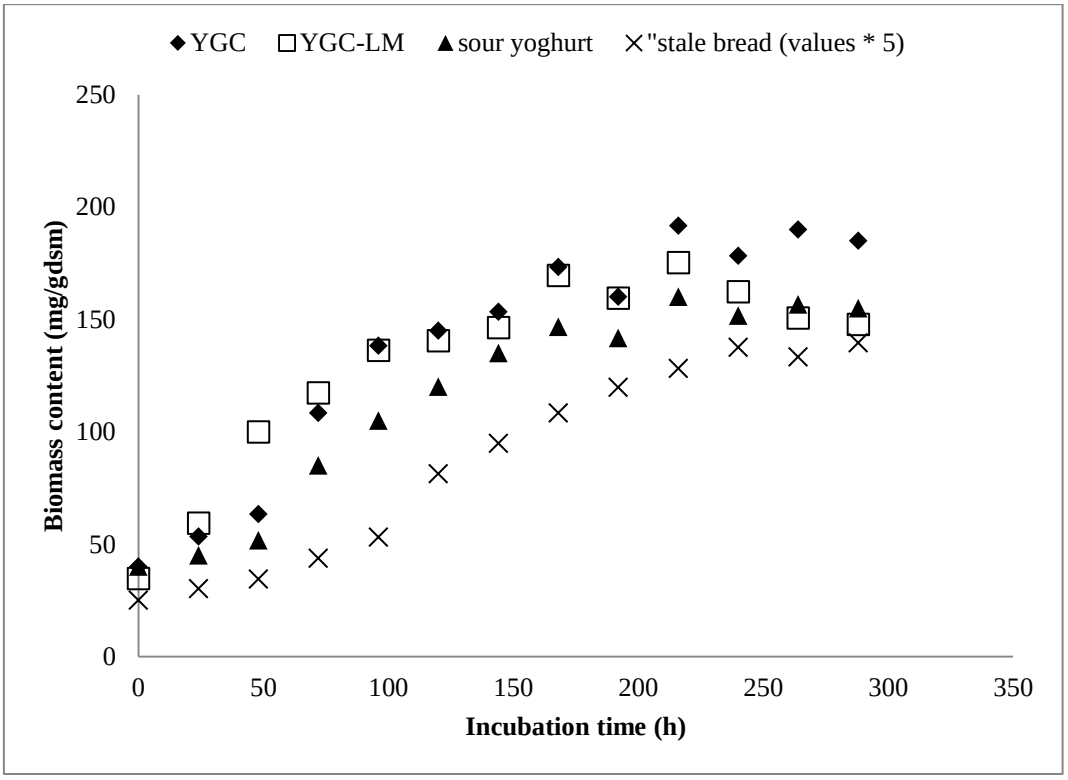

Figure 2. Biomass kinetics of *M. purpureus* grown on defined media (YGC and YGC-LM) and food residues (stale bread and sour yoghurt) following 12 days (value belongs to stale bread is multiplied with 5 to see the trend apparently)

Within three days rapid biomass formation of *M.purpureus* was obtained on YGC-LM plate as compared to YGC only. The relative biomass amount with YGC was 190 mg/gdsm, followed 175 mg/gdsm with YGC-LM at the highest levels as shown in Fig. 2. Litmus milk contains the carbohydrate lactose along with three main proteins, i.e. casein, lactalbumin and lactoglobulin. Tseng et al., 2000 [22] indicated higher protease activity and lower pigment yield in the medium with lactose replacing of glucose. This could increase the metabolic activities of the mycelia, and so the biomass yield was higher when compared with the YGC media. The relative biomass amount was decreased to 160 mg/gdsm with sour yoghurt, and 27.5 mg/gdsm with stale bread. Waste bread includes 60 % of starch and 8.9 % protein in dry base [23]. During growth, *M. purpureus* breaks down starch substrate into several metabolites, of which pigments are produced as secondary metabolites. In previous studies, the starch rich agricultural products were also studied as substrate for pigment production [24]. Lee et al., 1995 [25] used a tapioca starch for the *Monascus* pigment production and they reported that, 50 g/l starch in the medium was ideal dose to observe the highest culture growth rate and pigment yield of *Monascus*.

3.3. M. Purpureus Kinetics

The applicability of stale bread and sour yoghurt as food wastes as substrates for Monascus pigment production were evaluated in Figure 3.

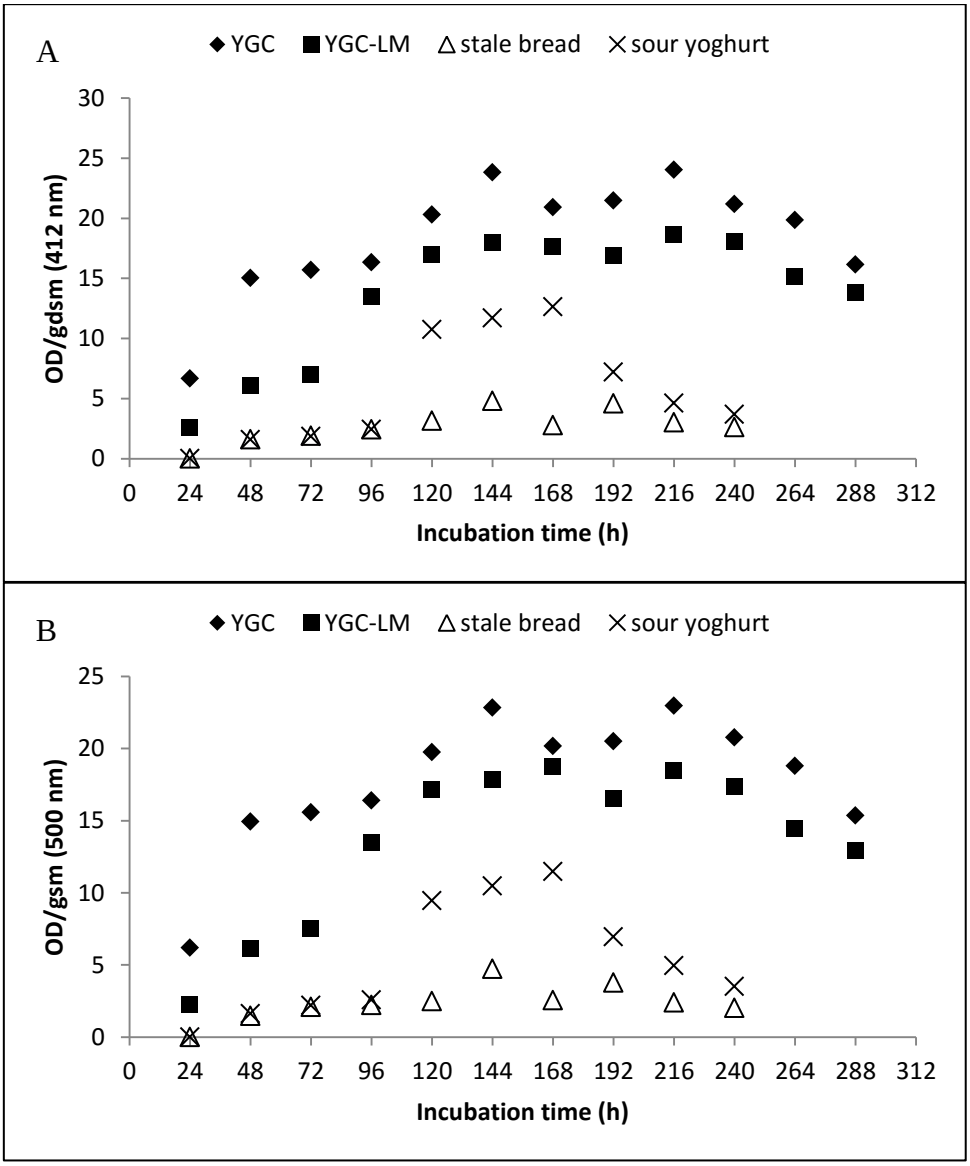

Figure 3. Growth and pigment yield of *Monascus purpureus* based on OD measurement at A: 412 nm and B:500 nm in various substrates

The comparisons of pigment production rates with defined medium were also performed. Spectral analysis indicated maximum absorbance at 412 nm and 500 nm were achieved when YGC was used. Since litmus milk favored proteolitic activity rather than pigment production, YGC-LM substrate yielded lower pigment yield than the YGC, as expected. Lower ethanol yield was also a good indication of this situation (Fig. 4).

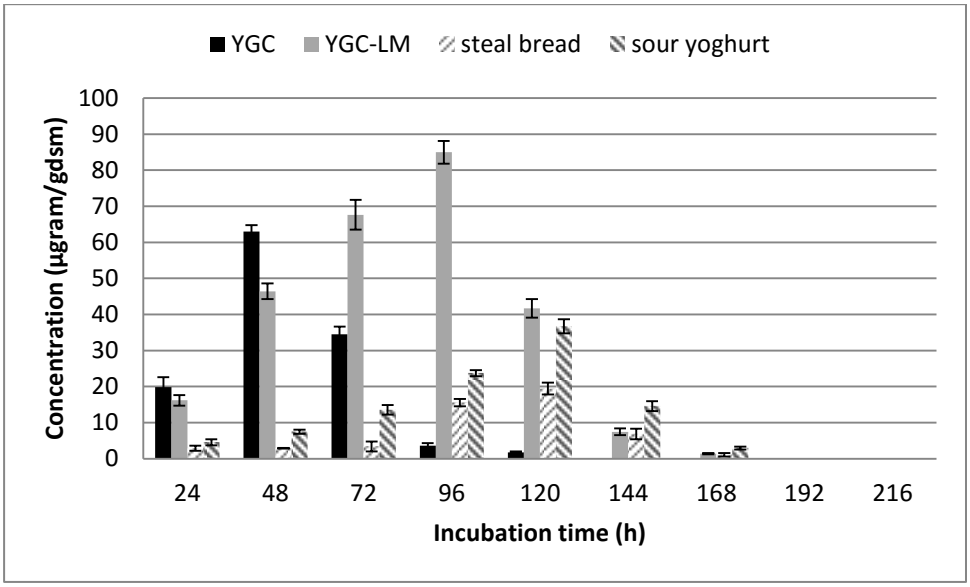

Figure 4. Ethanol production and consumption of *M.purpureus* grown on different substrates

In case of the food residues used as substrates, fermentation yielded red pigment concentration as 12.5 OD/gdsm for sour yoghurt and 4.8 OD/gdsm for stale bread, which were almost half and 1/6 of the highest pigment yield achieved with the defined media, respectively. In literature, for the other agro industrial wastes, highest pigment yield was achieved as; corn cob (25.4 OD/gdsm), coconut oil cake (0.12 OD/gdsm), tamarind seed powder (1.15 OD/gdsm), cassava flour (1.46 OD/gdsm), wheat bran (3.52 OD/gdsm), spent brewing grain (4.35 OD/gdsm), jackfruit seed powder (12.11 OD/gdsm) [25].

3.4. Ethanol Formation/Consumption during the Growth Kinetic

Fermentation studies showed that the final pigment yield correlated with the transient appearance of ethanol and its consumption. Many authors have reported on ethanol production in *Monascus sp.* both in submerged [26] and solid-substrate cultivation [27]. The levels of ethanol accumulated increased when the defined medium were used (Fig. 4). Since litmus milk supported to the metabolic activity, the biomass yield became higher, at the initial period of the fermentation (Fig. 2). Then, ethanol as a growth- associated metabolic product reached to the highest level as 84 µg / gdsm at 4 th day of the fermentation (Fig. 4). Lee et al., 2001 [28] also indicated that, glucose concentration was very important, because increasing glucose concentration increased both biomass and pigment production up to the 30 g/L. However, although ethanol accumulation favored MPs production as a secondary metabolite, lower pigment formation was detected when LM used in the media formula (Fig. 3). This indicated that different substrates yielded varying pigment and metabolite production.

3.5. HPLC Profile of monascus pigments

HPLC chromatogram of *Monascus* pigments at 238 nm is shown in Fig. 5.

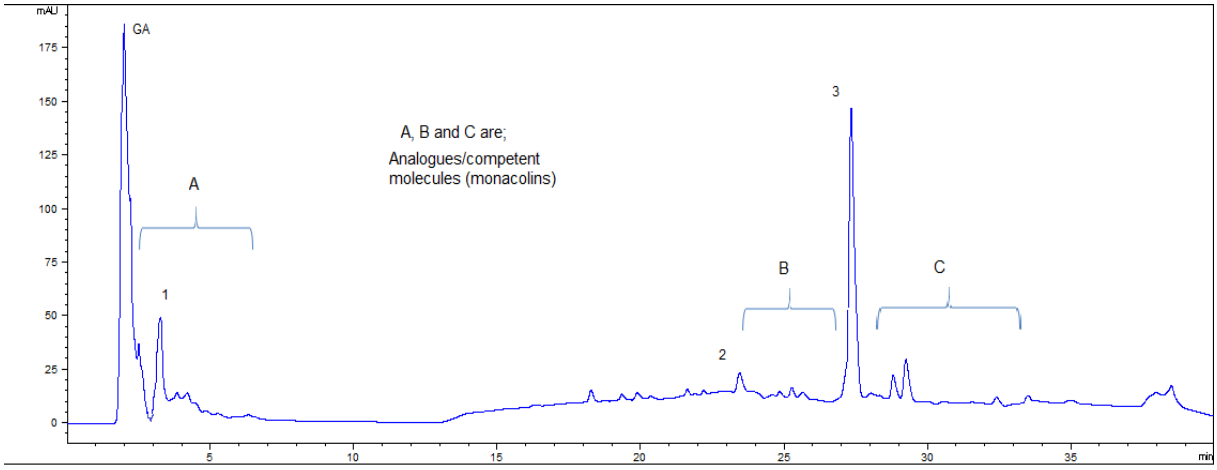

Figure 5. HPLC chromatogram of monascus metabolites; (detection at 238 nm); 1: monacolin K; 2: hydroxy acid form of lovastatin; 3: Lactone form of lovastatin ad their possible analogues/compotent molecules in fermentation media (shown as A,B, and C regions). Chromatogram of Monascus purpureus fermentation sample, which was taken over the YGC agar at the end of the fermentation, contains gallic acid (24 µg GA/mL) as an external standard

Due to the biosynthesis pathway of lovastatin and its analogues from acetate, these compounds were accumulated in the fermentation medium and resulted in complex environment. It has been reported that the presence of diene groups in lovastatin were responsible for this typical interference [19]. Some of the intermediates of lovastatin, such as monacolin J, X, L and M were also found to have maximum absorbance at 238 nm [29]. These compounds appear as probable interferences in the determination of MPs, such as lovastatin and monacolin (Regions A, B and C were signed on the chromatogram). However, relative comparative analysis was performed to evaluate the difference in metabolites of *Monascus*. A comparison was performed with the following formula;

$$GAEq.component = \frac{GA\ standart\ (\mu g/ml)}{peak\ area\ of\ GA} x peak\ area\ of\ component$$

where GAEq. is the Gallic acid equivalent.

Table 1 tabulated the level of lovastatin and their analogues determined in the fermentation environment prepared with different substrates.

Table 1. Substrate effects on the monascus pigments; comparative analysis based on the gallic acid (GA); where samples were taken at the end of the fermentation

Substrate	Monacolin K (GAEq.)	Hydroxy acid form Lovastatin (GAEq.)	Lactone form Lovastatin (GAEq)
YGC	8.78	2.44	25.44
YGC-LM	4.17	5.56	21.65
Sour yoghurt	2.11	3.17	16.41
Stale bread	5.58	1.04	21.48

Type, amount and yield of MPs differ due to the varying growth performances of *M.purpureus* on different substrates. Biosynthetic pathway also has a major role to determine the MPs composition. Furthermore, accumulation of certain components might eliminate to synthesize valuable bioactive molecules. As parallel to this observation, Xie et al., 2006 [30] reported the accumulation of monacolins especially, monacolin J, inhibited lovastatin biosynthesis.

3.6. Kinetic Model and Kinetic Parameters within Different Substrates

The non-linear fitting of kinetic equations was conducted with the experimental data and the kinetic parameters were determined using Matlab software (Fig. 6).

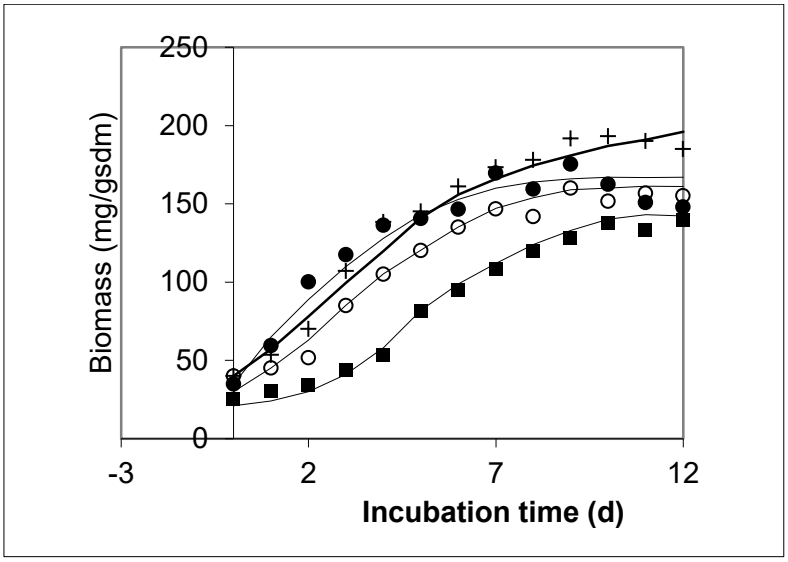

Figure 6. Model (—) and experimental data of biomass for different substrates; YGC (+); YGC-LM (•); sour yoghurt (o); stale bread (□).

The maximum specific growth rate, μ_{max} and K_s values were tabulated in Table 2. The constant K_s is the concentration of the rate-limiting substance when the specific rate of growth is equal to one-half of its maximum. It is also known as the Monod or saturation constant and shows the affinity of the organism for the growth-limiting substrate [20].

Table 2. Model parameters and correlation coefficients for different substrates used

Substrate	μ_{max}	K_s	R^2
YGC	0.412	0.78	0.979
YGC-LM	0.432	0.81	0.950
Sour yoghurt	0.406	0.64	0.986
Stale bread	0.387	0.67	0.996

Due to the different nutrient properties of substrates, they provided different microenvironments for the *M.purpureus* growth, metabolite and MPs production. Hence, the kinetic parameters were apparently different between all fermentation systems. As shown in the Table 1, the maximum specific growth rate (μ_{max}) was achieved highest when the YGC-LM used as a substrate. The metabolic function and ethanol formation also supported this high value. However, μ_{max} values of sour yoghurt and stale bread also very close to the

defined medium's values. This indicated that, food residues could effectively be used as substrates for the *M. purpureus* growth.

3. CONCLUSION

This research attempts to identify main stages of the *M.purpureous* adaptation, growth performance and link them to changes in Monascus metabolites during the fermentation period. Non-growth associated product formation was established and product formation was achieved after ethanol accumulation in the fermentation environment. According to the promising results achieved, *Monascus* pigment production by fermentation using complex media such as bread and yoghurt residues or wastes is feasible, economic and safe process. This process has the facility of converting no-value food wastes to the *Monascus* pigments and metabolites, which serve functional properties for food industry. Resultant *Monascus* pigments have great potential of replacing synthetic food colorants by these natural ones.

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