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ORIGINAL ARTICLE

Optimization of date syrup as a novel medium for lovastatin production by *Aspergillus terreus* ATCC 20542 and analyzing assimilation kinetic of carbohydrates

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Abstract

Lovastatin is a statin drug, which lowers cholesterol level in blood due to inhibition of (S)-3-hydroxy-3-methylglutaryl-CoA reductase. Date syrup is a rich medium for microbial growth and metabolite production. The main carbohydrates present in the date syrup are glucose and fructose. In this study, date syrup was used as a complex and bioresource medium for lovastatin production by *Aspergillus terreus* in the submerged cultivation. Optimization of the date syrup medium in order to achieve the highest titers of lovastatin and biomass was carried out. Four factors were studied by response surface methodology including concentration of date syrup carbohydrates, yeast extract concentration, pH, and rotation speed of the shaker. Optimal conditions for these factors found were as follows: concentration of date syrup carbohydrates, 64 g/l; yeast extract concentration, 15 g/l; pH, 6.5; and agitation speed, 150 rpm. It gave lovastatin concentration of 105.6 mg/l. Next, batch cultures in the optimal conditions were performed in a 2.5-l working volume bioreactor and led to the lovastatin titer of 241.1 mg/l during 12 days. *Aspergillus terreus* showed diauxic growth in the optimized medium with a shift from glucose to fructose assimilation during the run. Glucose and fructose assimilation kinetic parameters revealed that more lovastatin is produced during glucose assimilation, while more biomass was formed during fructose assimilation.

Keywords Lovastatin · Date syrup · *Aspergillus terreus* · Optimization · Fermentation

Introduction

Lovastatin ($C_{24}H_{36}O_5$) is a potent drug for lowering blood cholesterol (Porcel et al. 2006). Lovastatin is produced by microbial fermentation. Production can be carried out in either solid state or submerged cultures. The most eminent microorganisms used for lovastatin production are fungi like *Penicillium*, *Monascus*, and *Aspergillus*. But the current industrial production of lovastatin is mainly performed by *Aspergillus terreus* growing in submerged cultures (Goswami et al. 2012). Lovastatin is a secondary metabolite and mostly produce when growth was stopped then provided nitrogen deficiency and excess carbon is assured (Lopez et al. 2003).

The culture medium and their components have a significant influence on fungal growth and lovastatin production. (Lopez et al. 2003; Jia et al. 2009a). Many studies on lovastatin biosynthesis have been focused on the influence of media composition like carbon and nitrogen sources; elements like Zn, Fe, and Mg; B-group vitamins; inducers like polyketide antibiotics; as well as the impact of culture conditions such as oxygen saturation and pH (Szakács et al. 1998; Kumar et al. 2000; Hajjaj et al. 2001; Lai et al. 2002; Casas Lopez et al. 2004; Bizukojc et al. 2007; Lu et al. 2010). Jaberi Ansari et al. showed that carbon source, nitrogen source, C/N

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ratio, amount and type of inoculum, pH, minerals, and inducers are the most important factors affecting the production of lovastatin (Jaberi Ansari et al. 2016). In addition to the composition of media also morphology (or dimension) of the submerged hyphal growth pellet affect on lovastatin biosynthesis. Gao et al. investigated the relationship between pellet morphology and the production of itaconic acid in the *Aspergillus terreus* fungus and showed that increase of phosphorus concentration and temperature increases the diameter of the pellet and thus decreases the production of itaconic acid (Gao et al. 2014). The metabolic responses of *Aspergillus terreus* to stress conditions (low dissolved oxygen and pH with limited nitrogen and phosphate) in the two-phase fermentation were investigated by Songserm and et al. The fermentation kinetics suggested that itaconate production was suppressed under low dissolved oxygen (DO) concentrations (Songserm et al. 2018). Boruta and Bizukojc reviewed pH and fungal morphology on lovastatin production and concluded pH less than 7 and small pellet produce more lovastatin (Boruta and Bizukojc 2017). Rahim et al. used different carbon sources like D-galactose and D-ribose for production of lovastatin and their findings suggest that the production of lovastatin by *Aspergillus terreus* is highly influenced by the choice of carbohydrates that will shape the pellet morphology rather than the rate of carbohydrates metabolism (Rahim et al. 2017). Lu et al. showed that controlling morphology results in increasing of the production of such secondary metabolites, as itaconic acid, in the liquid culture (Lu et al. 2015). Anuradha et al. showed that pH level at 5.5, temperature at 28 °C, and stirring at 200 rpm improved the formation of pellet and the production of enzymes from *Aspergillus awamori* MTCC 9166 (Anuradha et al. 2014). Jaberi Ansari et al. reported factors effective in morphology of *Aspergillus terreus* in order to increase lovastatin production; the authors indicated that the type of carbon source has the greatest effect on morphogenesis of pellet, the formation of small pellets reduces the viscosity of the medium, increases the rate of oxygen transfer to microorganisms, and ultimately produces more lovastatin (Jaberi Ansari et al. 2017). Bizukojc and Gonciarz showed the correlation between pellet size, production of lovastatin, lactose consumptions, and oxygen availability in *A. terreus* (Bizukojc and Gonciarz 2015).

Atli et al. used Placket–Burman design for optimization glucose, peptone, and agitation speed in order to increase lovastatin production (Atlı et al. 2016). Dikshit and Tallapragada study optimization of lovastatin production from *Monascus sanguineus* under solid-state fermentation using response surface methodology (RSM); they optimized soybean concentration, $CaCl_2$ concentration, acetic acid concentration, and inoculum size for enhanced production of lovastatin (Dikshit and Tallapragada 2016). Suraiya et al. reported that brown seaweed was fermented in solid state by *Monascus purpureus* to maximize lovastatin production; the RSM was applied to optimize four different fermentation parameters: temperature (25–35 °C), time (10–20 days), glucose (0.1–1.5%), and peptone (0.1–0.7%) concentration. The predicted combination of process parameters yielding the highest rate of lovastatin production (13.40 mg/g) was obtained at a temperature of 25.64 °C, a fermentation time of 14.49 days, glucose concentration of 1.32%, and peptone concentration of 0.20%, with 92.85% validity. Among the studied factors, glucose, incubation time, and temperature most strongly influenced on lovastatin production (Suraiya et al. 2018). Mouafi et al. also applied response surface methodology for optimization of lovastatin production by *Aspergillus fumigatus* under solid-state fermentation using different agro-based wastes such as wheat bran (Mouafi et al. 2016). Recently, such cheap substrates as whey powder were added to the culture media for lovastatin production to improve the economical suitability of this production (Karthika et al. 2013). Kumar et al. used lignocellulose biomass as the carbon source for lovastatin production (Kumar et al. 2000). Sunflower oil, soybean oil, palm oil, and olive oil (Sripalakit et al. 2011) are complex substrates, which were utilized as alternative carbon sources for the production of lovastatin. These complex substrates allowed for the activation of the enzymes and genes involved in lovastatin production and hence increasing it compared to synthetic media (Lai et al. 2007). Chang et al. used response surface methodology to optimize rice powder, peptone, glycerin, and glucose for production of lovastatin by *Monascus ruber* (Chang et al. 2002). Results from Sripalakit et al. (2011) showed that complex media have a positive effect on lovastatin productivity. The worldwide production of dates is > 7.5 million tons in 2014 and Iran with > 1.15 million tones production, which ranked first among the Asian countries, in the same year (Karizaki 2017). Therefore, out of these complex substrates, date syrup is known to be an economically feasible bio-source for the conversion of carbohydrates to ethanol, citric acid, and α-glucoamylase because it is readily available and relatively cheap (Acourene and Ammouche 2012). Elsanhoty et al. used Placket–Burman design for optimization date waste to carotenoid production by *Lactobacillus plantarum* (Elsanhoty et al. 2012). Roukas and Kotzekidou reported that acid citric yield was increased by 50% compared to a control medium when the cultivation *Aspergillus niger* ATCC 9142 was performed on date syrup (Roukas and Kotzekidou 1997). Moreover, it was reported that the use of date syrup as a nutrient resulted in approximately sixfold decrease in cost of raw materials for schizophyllan production as compared to the conventional carbon sources (Jamshidian et al. 2016). Acourene and Ammouche concluded that date syrup could serve as a potential substrate for biomolecules production in biotechnology (Acourene and Ammouche 2012).

Date syrup was therefore considered for lovastatin production in this study for the first time, to our best knowledge, and this medium can be used as cheap and effective for industrial

applications. In order to examine this hypothesis, the concentration of date syrup (carbon source), the concentration of yeast extract (nitrogen source), pH, and agitation speed for lovastatin and biomass production were first optimized with the use of response surface methodology. Then in order to further improve lovastatin production, the optimal conditions were tested in a batch stirred tank bioreactor. Furthermore, carbohydrates assimilation kinetics of *Aspergillus terreus* was analyzed under these optimal conditions.

Materials and methods

Microorganism, chemicals, and inoculation

Date syrup from Kabkab Date variety (SARADIPOUR Bushehr, Iran) was purchased from a local market. All chemicals of analytical grade used in this study were purchased from a Merck (Germany) representative. Lovastatin standard was kindly provided from the Osveh pharmaceutical factory (Tehran, Iran). *Aspergillus terreus* ATCC 20542 (imported from Belgium Culture Collection) was used in this study. After inoculation from the main slant, the culture was maintained on Petri dishes containing potato dextrose agar (PDA). The dishes were incubated at 28 °C for 5 days and subsequently stored at 5 °C. The suspension of spores for inoculation was prepared by washing the Petri dish cultures with a sterile aqueous solution of 0.5% Tween® 80. The resulting suspension was precipitated by centrifuging in (~ 2800×*g*, 5 min) and the pellet was resuspended in sterile distilled water. The initial spore concentration for inoculation was adjusted spectrophotometrically at 360 nm (NanoDrop 2000c, USA) to 10^7 spores/ml. For spores determination, a standard curve was used to correlate optical density and direct spore count (Lopez et al. 2003).

Response surface methodology

The experimental design formulated according to RSM was used to optimize the medium for lovastatin production. This method was applied to identify the optimal conditions for lovastatin production. Central composite design (CCD) is a RSM model, which involves a full second-order polynomial model. From preliminary study, four variable parameters including concentration of date syrup carbohydrates (A), yeast extract concentration (B), pH (C), and rotation speed of the shaker (D) were selected. Range of these parameters for each parameter was selected according to the outstanding article reports. These amount for date syrup carbohydrate were 48 to 112 g/l, for yeast extract concentration were 0 to 20 g/l, for pH were 5.5 to 9.5, and for agitation were 100 to 300 rpm. Variables were tested at five levels, namely higher (+2), high (+1), middle (0), low (−1), and lower (−2). Thirty experiments were carried out. Results were analyzed by DESIGN EXPERT 10.0.7 software (Statease, USA). The relative effects of two variables on the response were identified from 3D plots.

Cultivation conditions

Medium composition

Date syrup was used as a carbon source; for eliminating the difference between date syrup batches, all date syrup prepared from one batch of kabkab date during the experiments (Bushehr, Iran) was diluted by mixing with an appropriate volume of deionized water in order to obtain certain carbohydrates concentration; yeast extract was used as a nitrogen source, and in the end of preparation, pH was adjusted.

Shake flask cultivation

Date syrup based cultivations were carried out in 250-ml Erlenmeyer flasks containing 50 ml media. These flasks were inoculated with 2 ml of 10^7 spores/ml at 28 °C for 12 days on a rotary shaker at a given rotation speed, according to the experimental design.

Bioreactor cultivation

Optimal conditions established upon RSM experiments were used to make batch experiments in a 2.5-l total volume stirred tank bioreactor (SabaFerm 110, Iran).

The culture conditions were as follows. Agitation was initially set at 150 rpm and aeration rate at 1 vvm and 70% oxygen saturation level. The cultivation at 28 °C lasted for 12 days. Preculture for inoculation of the stirred tank was previously grown in flasks 2 ml of 10^7 spores/ml in 50 ml date syrup medium in 250-ml Erlenmeyer at 28 °C and at 150 rpm for 1 day.

Analytical methods

Glucose, fructose, and sucrose carbohydrates detection

Date syrup carbohydrates were determined by HPLC in order to find out the amount of each carbohydrates in date syrup and determine how much of it can be added for each experiment. The standard solutions of carbohydrates were prepared with 2 g of glucose, fructose, and sucrose per 100 ml distilled water. All standard solutions were diluted with distilled water and filtered through a 0.45-μm filter membrane (No. 1; Whatman, Maidstone, Kent, UK) prior to HPLC-analysis. Deionized water and acetonitrile (HPLC grade) were used as mobile phase; the ratio of acetonitrile and the deionized water used was (80:20 *v*/*v*). The HPLC system for carbohydrate measurement

was an Agilent 1100 with diode array detector. UV detection was made at 195 nm. The flow rate was set to 0.6 ml/min and injection volume was of 20 μl. The column used was Supelco Kromasil NH_2 column (250 mm × 4.5 mm, 5 μm) (Rahman et al. 2008). Column temperature was 30 °C.

Analysis of the mineral elements

Determination of the microelements in the date syrup was carried out after their extraction in deionized water. Dilution to achieve the desired range of calibration for each element was made with deionized water. Buck scientific atomic absorption spectroscopy (AAS) was used for the measurement of 30 elements (Salman et al. 2011).

Determination of fat, protein, and dietary fiber

Total dietary fibers of date syrup were determined according to the AOAC enzymatic-gravimetric method of Prosky et al. (1988).

Total protein and fat content were measured according to the procedure cited elsewhere (Assirey 2015).

Biomass assay

Sampled fungal suspensions were filtered through a 0.45-μm filter membrane (No. 1; Whatman, Maidstone, Kent, UK) and washed twice with distilled 50 ml water. It was then dried at 105 °C for 2 days for the measurement of dry weight (DW).

Lovastatin determination

A 5 ml of broth was sonicated, adjusted to pH 3.0 using HCl 1 N, and extracted by adding twice the volume of ethyl acetate into each samples at ambient temperature for 18 h. Before HPLC measurements, 5 ml of extract was filtered through a 0.45-μm membrane in order to filter the probably debris. Then supernatant was collected, lactonized with 5 ml 1% *v*/*v* trifluoroacetic acid (in water) in order to stabilized lovastatin (lovastatin have two forms: acidic and lactonized) for 1 h on shaker at 100 rpm. Finally, 5 ml acetonitrile was added to pervious medium and shook at 100 rpm for 1 day, then acetonitrile solution was separated and 20 μl of this solution was injected to HPLC for analysis (Su et al. 2003). The procedure given by Samiee et al. for HPLC analysis was slightly modified as follows. Lovastatin was determined by HPLC (SHIMADZU, Japan) using 250 × 4.6 mm ID Lichrosper® 100 C_{18} column, 5 μm particle size. Acetonitrile in water acidified (0.1%) with orthophosphoric acid (65:35 *v*/*v*) was used as the mobile phase. Flow rate of the mobile phase was maintained at 1.5 ml/min and detection was carried out by a UV detector at 238 nm (Samiee et al. 2003). The detection of lovastatin was conducted at room temperature with an injection volume of 20 μl. Authentic lactonized standards (Osveh Company, Iran) of lovastatin were used for calibration and further identification.

Calculation of yield and kinetic parameters

Three yield coefficients Y_{xp}, Y_{sp}, Y_{sx}, and biomass-specific growth rate (μ) were calculated in order to define which times and which types of carbohydrates were suitable for lovastatin biosynthesis and biomass production. These calculations were based on the difference of each two consecutive values of concentrations.

$Y_{xp}(mg/gDW) = \Delta c_p/\Delta c_x$; yield coefficient of product (lovastatin) on biomass
$Y_{sp}(mg/g) = \Delta c_p/\Delta c_s$; yield coefficient of product (lovastatin) on substrate (glucose or fructose)
$Y_{sx}(gDW/g) = \Delta c_s/\Delta c_x$; yield coefficient of biomass on substrate (glucose or fructose)
$\mu(1/h) : \mu = 1/c_x.(\Delta c_x/\Delta t)$ Specific growth rate

Each experimental analysis is repeated three times, and the results are average of them.

Results and discussion

Date syrup composition

Upon HPLC, it was found that glucose and fructose percentages in the date syrup were 37 and 43%, respectively. Table 1 shows the experimental results of date syrup minerals determination by atomic absorption. The highest concentrations in date syrup were found for K 6820.00 ppm, Na 1900.82 ppm, Ca 1368.24 ppm, and Mg 544.82 ppm.

Optimization by RSM for biomass formation

Having learned the concentration of main carbohydrates and mineral elements, date syrup-based medium was optimized for *A. terreus* growth and lovastatin production. CCD was employed according to the combination of variables given in

Table 1 Concentration of carbohydrates and mineral elements in date syrup

Glucose percentage (W/V)		Fructose percentage (W/V)		Sucrose percentage (W/V)		Fat percentage (W/V)		Dietary fiber percentage (W/V)		Protein percentage (W/V)	
37% ± 0.7		43% ± 1		3% ± 0.3		0.3% ± 0.0		7% ± 0.4		2% ± 0.1	
Ag (ppm)	Al (ppm)	Zr (ppm)	As (ppm)	B (ppm)	Ba (ppm)	Be (ppm)	Ca (ppm)	V (ppm)	Cd (ppm)		
0.02 ± 0.0	3.91 ± 0.57	0.08 ± 0.04	2.76 ± 0.77	10.14 ± 1.62	0.52 ± 0.1	0.00 ± 0.0	1368.24 ± 68.2	0.04 ± 0.01	0.00 ± 0.0		
Co (ppm)	Sn (ppm)	Cu (ppm)	Fe (ppm)	Hg (ppm)	K (ppm)	La (ppm)	Si (ppm)	Mg (ppm)	Mn (ppm)		
0.03 ± 0.02	534.29 ± 33.2	1.24 ± 0.14	20.06 ± 1.08	1.21 ± 0.18	6820.00 ± 470.32	0.51 ± 0.087	295.93 ± 5.1	544.82 ± 22.02	3.53 ± 1.15		
Y (ppm)	Zn (ppm)	Na (ppm)	Pb (ppm)	Ti (ppm)	P (ppm)	Sr (ppm)	Sb (ppm)	Sc (ppm)	Se ppm))		
0.02 ± 0.01	1.47 ± 0.64	1900.82 ± 34.21	0.00 ± 0.0	0.29 ± 0.14	583.22 ± 14.64	22.82 ± 1.7	0.32 ± 0.17	36.74 ± 6.2	2.38 ± 0.49		

the design table (Table 2), and the results are given in tree last columns of Table 2.

Regarding biomass, the results showed that concentration of date syrup carbohydrates (A), yeast extract concentration (B), pH (C) and square effect of yeast extract (B^2), pH (C^2), and stirring speed (D^2) were significant at 95% in the production of biomass ($p < 0.05$) while stirring speed (D), AB, AC, and BC were not significant. The adequacy of the model (Eq. 1) was checked with the use of analysis of variance (ANOVA). The model was relevant as ANOVA gave a p value less than 0.05. The R^2 value equal to 94.6% confirmed model adequacy, showing the high correlation between experimental and predicted data values.

$$\begin{aligned}\text{Biomass (gDW/l)} = {} & +28.12\text{-}1.11 \cdot A + 6.46 \cdot B + 1.59 \\ & \cdot C\text{-}0.34 \cdot D\text{-}0.20 \cdot AB + 0.36 \cdot AC \\ & + 0.42 \cdot AD + 0.22 \cdot BC + 0.077 \\ & \cdot BD\text{-}0.13 \cdot CD\text{-}0.79 \cdot A^2\text{-}1.78 \\ & \cdot B^2\text{-}0.97 \cdot C^2\text{-}1.65 \cdot D^2 \qquad (1)\end{aligned}$$

According to Table 2, the maximum biomass amount (37.24 gDW/l) was obtained for experiment 24, namely under the following conditions: concentration of date syrup carbohydrates, 80 g/l; concentration of yeast extract, 20 g/l; pH, 7.5, and agitation speed, 200 rpm, while the minimum biomass amount was (5.9 gDW/l) obtained for run 23, namely for concentration of date syrup carbohydrates 80 g/l, without yeast extract, pH 7.5, and agitation speed at 200 rpm.

Contour plots

The optimum level of each variable and the effect of their interactions on biomass production as a function of two variables were studied by plotting contour plots by holding the other two factors constant at defined levels. The hold values of the concentration of date syrup carbohydrates, yeast extract, pH, and rotation speed were 64 g/l (− 1), 15 g/l (+ 1), 7.5 (0), and 150 rpm (− 1) respectively. The interaction effect between pairs of variables on biomass was well understood by the contour plots represented in Fig. 1. Figure 1a illustrates the relation between concentration of date syrup carbohydrates and yeast extract concentration on biomass production and showed that maximum biomass amounts were observed at low levels of the concentration of date syrup carbohydrates and higher levels of yeast extract. When the concentration of date syrup carbohydrates and yeast extract concentration were 64 g/l (− 1) and 20 g/l (+ 2) respectively, high biomass production was observed (Fig. 1)a. Figure 1b shows the interaction effect of concentration of date syrup carbohydrates and pH on

Table 2 Response surface method design with results observed for lovastatin biosynthesis, biomass production, and lovastatin production yield in date syrup medium in flask

Standard order	Blocks	(A) Concentration of date syrup carbohydrates (g/l)	(B) Yeast extract concentration (g/l)	(C) pH	(D) RPM	Lovastatin (mg/l) actual	Biomass (gDW/l) actual	Yield (mg/gDW) actual
1	1	64 (− 1)	5.00 (− 1)	6.50 (− 1)	150 (− 1)	24.60	15.54	1.58
2	1	96 (+ 1)	5.00 (− 1)	6.50 (− 1)	150 (− 1)	15.30	15.38	0.99
3	1	64 (− 1)	15.00 (+ 1)	6.50 (− 1)	150 (− 1)	105.60	29.64	3.56
4	1	96 (+ 1)	15.00 (+ 1)	6.50 (− 1)	150 (− 1)	18.75	22.14	0.84
5	1	64 (− 1)	5.00 (− 1)	8.50 (+ 1)	150 (− 1)	15.30	18.84	0.81
6	1	96 (+ 1)	5.00 (− 1)	8.50 (+ 1)	150 (− 1)	14.40	17.06	0.84
7	1	64 (− 1)	15.00 (+ 1)	8.50 (+ 1)	150 (− 1)	77.70	30.48	2.54
8	1	96 (+ 1)	15.00 (+ 1)	8.50 (+ 1)	150 (− 1)	13.80	30.14	0.45
9	1	64 (− 1)	5.00 (− 1)	6.50 (− 1)	250 (+ 1)	22.20	15.02	1.47
10	1	96 (+ 1)	5.00 (− 1)	6.50 (− 1)	250 (+ 1)	18.30	14.9	1.22
11	1	64(− 1)	15.00 (+ 1)	6.50 (− 1)	250 (+ 1)	26.55	27.5	0.96
12	1	96 (+ 1)	15.00 (+ 1)	6.50 (− 1)	250 (+ 1)	21.00	26	0.80
13	1	64 (− 1)	5.00 (− 1)	8.50 (+ 1)	250 (+ 1)	13.80	19.38	0.71
14	1	96 (+ 1)	5.00 (− 1)	8.50 (+ 1)	250 (+ 1)	15.03	16.62	0.90
15	1	64 (− 1)	15.00 (+ 1)	8.50 (+ 1)	250 (+ 1)	21.60	28.94	0.74
16	1	96 (+ 1)	15.00 (+ 1)	8.50 (+ 1)	250 (+ 1)	12.30	30.3	0.40
17	1	80 (0)	10.00 (0)	7.50 (0)	200 (0)	85.03	29.1	2.92
18	1	80 (0)	10.00 (0)	7.50 (0)	200 (0)	63.84	24	2.66
19	1	80 (0)	10.00 (0)	7.50 (0)	200 (0)	86.78	29.04	2.98
20	1	80 (0)	10.00 (0)	7.50 (0)	200 (0)	85.38	28.24	3.02
21	2	48 (− 2)	10.00 (0)	7.50 (0)	200 (0)	45.84	29	1.58
22	2	112 (+ 2)	10.00 (0)	7.50 (0)	200 (0)	9.60	22.04	0.43
23	2	80 (0)	0.00 (− 2)	7.50 (0)	200 (0)	6.00	5.9	1.01
24	2	80 (0)	20.00 (+ 2)	7.50 (0)	200 (0)	36.30	37.24	0.97
25	2	80 (0)	10.00 (0)	5.50 (− 2)	200 (0)	9.30	21.68	0.42
26	2	80 (0)	10.00 (0)	9.50 (+ 2)	200 (0)	7.80	27.94	0.27
27	2	80 (0)	10.00 (0)	7.50 (0)	100 (− 2)	24.00	24	1
28	2	80 (0)	10.00 (0)	7.50 (0)	300 (+ 2)	11.10	20.16	0.55
29	2	80 (0)	10.00 (0)	7.50 (0)	200 (0)	85.78	28.54	3.00
30	2	80 (0)	10.00 (0)	7.50 (0)	200 (0)	82.54	28.72	2.87

biomass production. The maximum biomass was obtained at low level of the concentration of date syrup carbohydrates, 64 g/l (− 1) and high level of pH, 8.5 (+ 1). Interaction effects between yeast extract and pH are shown in Fig. 1c and maximum biomass concentration was achieved when yeast extract concentration at higher level, 20 g/l (+ 2) and high level of pH, 8.5 (+ 1). Due to p values higher than 0.05, interactions with agitation speed were not significant and hence were not displayed.

Medium optimization by RSM for lovastatin production

The adequacy of the model (Eq. 2) was checked using ANOVA and the corresponding results for lovastatin formation are given in Table 2, showing that the concentration of date syrup carbohydrates (A), yeast extract concentration (B), and the agitation speed (D) were significant ($p < 0.05$) while pH (C) was not significant. All square effect of parameters, as well as interaction effects between concentration of date syrup carbohydrates and yeast extract concentration (AB), concentration of date syrup carbohydrates and agitation speed (AD), and yeast extract and agitation speed (BD), showed a significant effect on lovastatin production with p values lower than 0.05. The R^2 value, 92.9%, indicated on the high correlation between experimental and predicted values; this implies that 92.9% of lovastatin production can be attributed to the independent variables, while only 7.1% of the total variation was not explained by the model (Eq. 2).

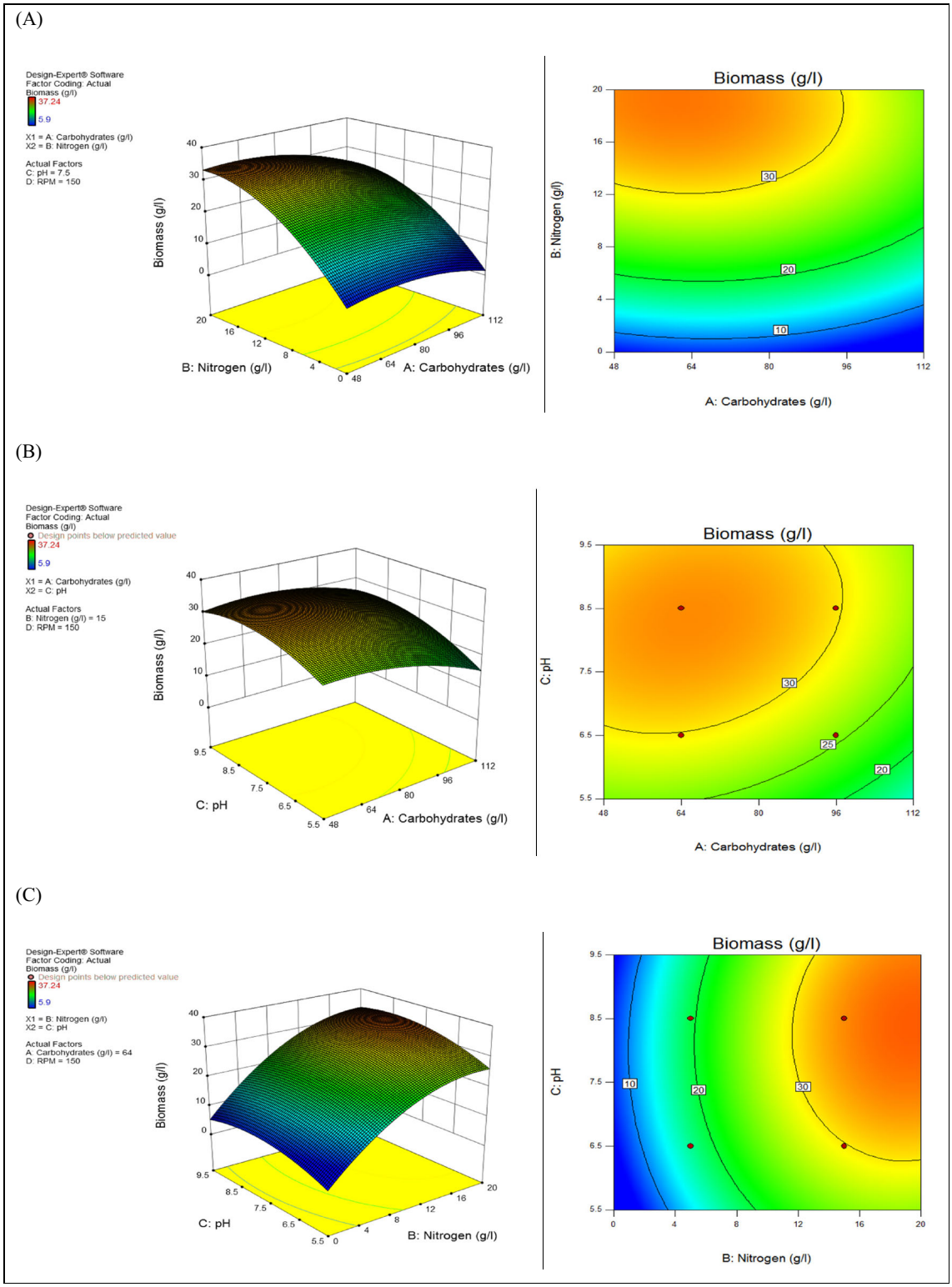

Fig. 1 Response surfaces and contour plots showing the interactions of every two pairs of effective variable parameters on maximum biomass formation: **a** the interaction of concentration of date syrup carbohydrates and yeast extract concentration. Maximum biomass (number 24 expriment (37.24 gDW/l)) formation was observed at the low level of concentration of date syrup carbohydrates (64 g/l (− 1)) and higher level of yeast extract (20 g/l (+ 2)). **b** The interaction of date syrup and pH, the figure was illustrated that the maximum biomass was obtained at low level of concentration of date syrup carbohydrates (64 g/l (− 1)) and high level of pH (8.5 (+ 1)). **c** The interaction of yeast extract concentration and pH; maximum biomass concentration was achieved when yeast extract concentration at higher (20 g/l (+ 2)) and pH value at high level (8.5 (+ 1))

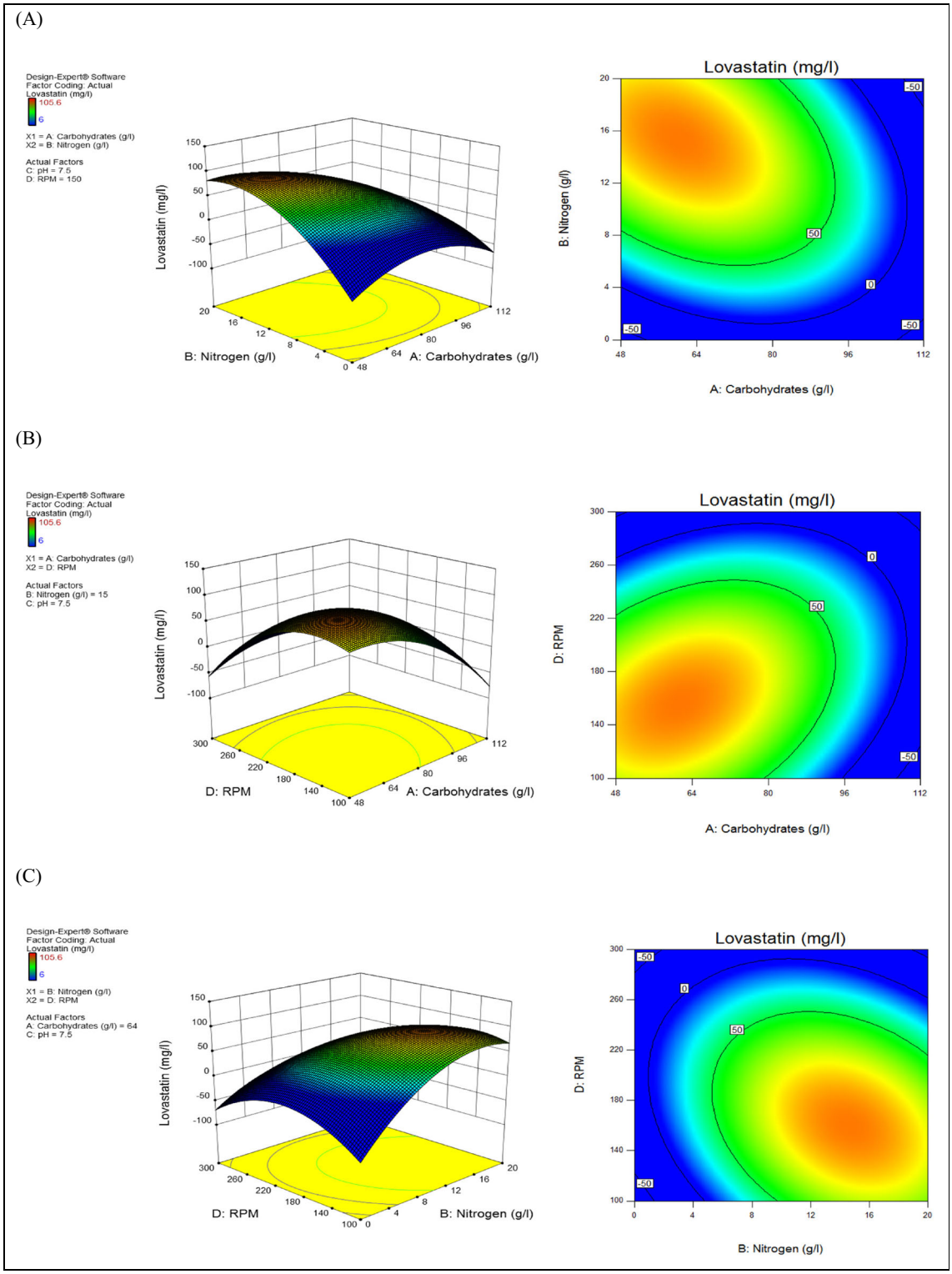

Fig. 2 Response surfaces and contour plots showing the interactions of every two pairs of effective variable parameters on maximum lovastatin formation. **a** The interaction of concentration of date syrup carbohydrates and yeast extract concentration, maximum lovastatin concentration (number 3 expriment (105.6 mg/l)) was observed at the low level of concentration of date syrup carbohydrates (64 g/l (− 1)) and high level of yeast extract (15 g/l (+ 1)). **b** The interaction of date syrup and rotation speed. It was illustrated that the maximum lovastatin formation was obtained at low levels of both concentration of date syrup carbohydrates (64 g/l (− 1)) and rotation speed (150 rpm (− 1)). **c** The interaction of yeast extract concentration and rotation speed, maximum lovastatin concentration was achieved by yeast extract concentration at high level (15 g/l (+ 1)) and rotation speed at low level (150 rpm(− 1))

$$\text{Lovastatin concentration (mg/l)} = +80.55\text{-}10.46 \cdot A + 9.128 \cdot B\text{-}2.79 \cdot C\text{-}6.69 \cdot D\text{-}9.55 \cdot AB + 2.05 \cdot AC + 8.96 \cdot AD\text{-}1.54 \cdot BC\text{-}8.38 \cdot BD + 1.11 \cdot CD\text{-}1204 \cdot A^2\text{-}13.68 \cdot B^2\text{-}16.83 \cdot C^2\text{-}14.58 \cdot D^2 \quad (2)$$

According to Table 2, maximum lovastatin concentration was (105.6 mg/l), achieved in run 3, namely for 64 g/l concentration of date syrup carbohydrates, 15 g/l yeast extract concentration, pH 6.5, and agitation speed 150 rpm, while minimum lovastatin production, (6 mg/l), was observed in run 23, namely for 80 g/l concentration of date syrup carbohydrates, without yeast extract, pH 7.5, and agitation speed 200 rpm.

Contour plots

As above it was made for biomass, the similar analysis with the use of contour plots was performed for the optimum lovastatin concentration. The optimum level of each variable and the effect of their interactions on lovastatin production as a function of two variables were studied by plotting contour plots while keeping the other two factors constant at given levels. The hold values of the concentration of date syrup carbohydrates, yeast extract, pH, and agitation speed were 64 g/l (− 1), 15 g/l (+ 1), 7.5 (0), and 150 rpm (− 1), respectively. The interaction effects between pairs of variables on lovastatin formation were shown by the contour plots illustrated in Fig. 2. Figure 2a illustrates the relation between concentration of date syrup carbohydrates and yeast extract concentration on lovastatin production, showing that maximum lovastatin concentration (105.6 mg/l) was observed at a low level of the concentration of date syrup carbohydrates and a high level of yeast extract, sixfold (− 1) and 15 g/l (+ 1) respectively. Figure 2b shows the interaction effect of the concentration of date syrup carbohydrates and agitation speed on lovastatin production, showing maximum formation at low levels of both the concentration of date syrup carbohydrates, 64 g/l (− 1), and the agitation speed 150 rpm (− 1). Interaction effects between yeast extract concentration and agitation speed (Fig. 2c) showed that maximum lovastatin concentration was achieved at a high level of yeast extract, 15 g/l (+ 1), and a low agitation speed, 150 (− 1).

Overall lovastatin production yield (total amount of lovastatin production per dried weight of biomass during the cultivation time) for all runs were calculated and collected in Table 2, showing maximum yield, 3.56 mg/gDW, for run 3, namely for 64 g/l concentration of date syrup carbohydrates (− 1), 15 g/l yeast extract (+ 1), pH 6.5 (− 1), and 150 rpm agitation speed (− 1). These culture conditions were ultimately selected for the batch cultivation in a stirred tank bioreactor.

Fermentation in stirred tank bioreactor

Batch culture was run for 12 days leading to lovastatin and biomass concentrations of (241.1 mg/l) and (43 gDW/l) respectively (Fig. 3). As seen in Fig. 3, fast biomass growth was observed during the first 2 days, followed by a stationary growth phase until 6 day. Second growth phase lasted from 6 to 12 day, illustrating diauxic growth of *A. terreus* in date syrup. It was in agreement with temporal changes of glucose and fructose concentrations, being the main carbohydrates in date syrup. Indeed, glucose consumption was observed during the first 6 days; later on, fructose assimilation was started.

It is seen in in Fig. 3 that more biomass was produced during fructose assimilation compared to glucose assimilation, 28.1 and 14.8 gDW/l respectively, while the amounts of each carbohydrate in date syrup were roughly similar and approximately was 30 g/l for glucose and 37 g/l for fructose. Regarding lovastatin production, more lovastatin was produced during glucose assimilation compared to fructose assimilation and the maximum lovastatin production for glucose and fructose utilization periods were 143.91 and 97.17 mg/l, respectively (Fig. 3).

Yield and kinetic aspects of glucose and fructose assimilation by *Aspergillus terreus*

Several yield and kinetic parameters including yield of product (lovastatin) on biomass (Y_{xp}), yield of product (lovastatin) on substrate (glucose or fructose) (Y_{sp}), and yield of biomass on substrate (glucose and fructose) (Y_{sx}) were calculated. Also the specific biomass growth rate (μ) for the periods of glucose and fructose consumption was measured. They were all collected in Table 3.

The product on biomass yield (Y_{xp}) showed a maximum value after 4 days of culturing on glucose assimilation equal to 66.91 mg/gDW, and the overall (Y_{xp}) in glucose and fructose assimilation were 9.69 and 3.45 mg/gDW, respectively.

The product on substrate yield (Y_{sp}) showed also a maximum value after 4 days of culturing on glucose assimilation was 33.45 mg/g, and the overall (Y_{sp}) in glucose and fructose assimilation were 5.8 and 3.57 mg/g respectively. Contrarily, the biomass on substrate yield (Y_{sx}) showed a maximum value after 12 days of culturing on fructose assimilation was 1.11 g/g. The overall (Y_{sx}) in glucose and fructose assimilation were 0.59 and 1.03 g/g respectively.

Specific biomass growth rate calculation based on Monod model revealed lower growth on glucose than that on fructose. The highest value of specific growth rate during the glucose was 0.102 h^{-1} and this value during fructose consumption period was 0.195 h^{-1}.

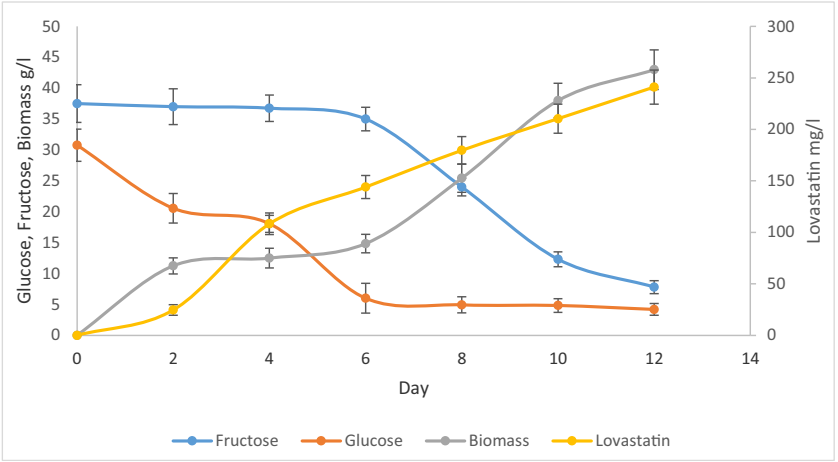

Fig. 3 Biomass growth, lovastatin biosynthesis, and substrate consumption by *Aspergillus terreus* with the use of the optimum date syrup-based medium in a batch stirred tank bioreactor

Date syrup is a cheap substrate, rich in carbohydrates (glucose and fructose) and mineral elements. Glucose was shown to be a suitable carbon source for lovastatin production and in the presence of yeast extract fructose was shown to be the most efficient carbon source for *A. terreus* growth (Lopez et al. 2003). Complex media like date syrup contain essential elements for microbial production of such metabolites as lovastatin. For instance, date syrup contains high amounts of such divalent metal ions as Zn or Fe, which have an impact on lovastatin production by regulating the action of key enzymes such as LovD or LovF in lovastatin biosynthesis. Furthermore, such elements as Fe, Ca, Zn, Mg, and Mn promote cell growth (Jia et al. 2009a).

RSM optimization showed that lovastatin biosynthesis was optimal 105.6 mg/l in run 3 and further improved during batch culture in larger scale of a stirred tank bioreactor (3 l), reaching 241.1 mg/l. This finding, according to Shindia, confirmed that media containing glucose and yeast extract stimulate lovastatin biosynthesis leading to its high productivities (Shindia 2001). Also our results in date syrup medium compared the use of lactose about 70 mg/l reported by Bizukojc et al. in 7 days in stirred tank bioreactor (Bizukojc and Ledakowicz 2008) and 200 mg/l in 9 days in bubble column were better (Rodriguez Porcel et al. 2007).

Our findings showed that lovastatin biosynthesis and biomass production were not impressive at high concentration of date syrup carbohydrates; this result can be due to the natural existence of such organic acids as acetic, propionic, butyric, and formic in date syrups. They had an inhibitory effect on lovastatin biosynthesis and biomass production (Al-Taweil et al. 2015). On the other hand, the concentration of date syrup and the amount of elements increased with increasing date syrup concentration. Some of them can probably reach their inhibitory levels. It was reported by other researchers who claimed about inhibitory effect of high amounts of mineral elements (Jia et al. 2009a). This study also showed that in contrast to the previous findings with other carbon substrates, in the date syrup-based media, high concentrations of yeast extract, namely nitrogen, had a positive effect on the production of lovastatin (Lopez et al. 2003; Bizukojc and Ledakowicz 2008). This study showed that date syrup did not contain enough utilizable nitrogen for growth of *Aspergillus terreus* and hence maximum biomass and lovastatin production were achieved at relatively high amount of yeast extract. It is in agreement with some literature sources, in which the utilization of whey powder for production of lovastatin was reported (Karthika et al. 2013).

This results showed that pH had a little effect on lovastatin biosynthesis because date syrup is a buffered medium and after a while, pH of medium reached its initial natural acidic pH. Hence pH level can influence biomass growth to the higher extent. At the beginning of the experiments, pH was stable but after a while, when lovastatin biosynthesis was started, pH gradually went down reaching the constant value

Table 3 Showing fermentation kinetic and specific growth rate of *Aspergillus terreus* in optimum date syrup medium in stirred tank bioreactor

	Glucose			Fructose			Glucose total (first 6 days)	Fructose total (second 6 days)
Days	2	4	6	8	10	12	–	–
Y_{xp} (mg/gDW)	2.18 ± 0.4	66.91 ± 3	15.17 ± 0.9	3.36 ± 0.5	2.45 ± 0.85	6.15 ± 1.05	9.69 ± 0.87	3.45 ± 0.3
Y_{sp} (mg/g)	2.40 ± 0.4	33.45 ± 2.4	2.95 ± 0.55	3.24 ± 0.71	2.62 ± 0.61	6.83 ± 0.39	5.80 ± 0.55	3.57 ± 0.32
Y_{sx} (gDW/g)	1.1 ± 0.21	0.5 ± 0.01	0.19 ± 0.05	0.96 ± 0.11	1.07 ± 0.25	1.11 ± 0.17	0.59 ± 0.08	1.03 ± 0.1
Specific growth rate (h^{-1})	0.234 ± 0.061	0.026 ± 0.0	0.048 ± ±	0.220 ± 0.04	0.261 ± 0.06	0.104 ± 0.0	0.102 ± 0.06	0.195 ± 0.02

at the level of 4. Upon literature data, it was found that the highest lovastatin production can be achieved at initial pH value of 6.5 (Bizukojc and Ledakowicz 2008; Bizukojc et al. 2012).

Agitation speed had a negative effect on lovastatin production. It was the best at low agitation speed equal to 150 rpm; it was like that due to probably a damaging effect of high agitation conditions on desired mycelial morphology (pellets). It was previously found that morphogenesis of fungus had an important effect on secondary metabolites production (Porcel et al. 2005). Although increasing the agitation speed improves aeration (Gbewonyo et al. 1992) but filamentous fungi pellets can be destroyed at high agitation conditions which caused high shear stress and breaking fungal mycelium. That is why lovastatin biosynthesis was reduced in high agitation in this study.

It was previously found that lovastatin production increased in the stirred tank bioreactors due to better aeration and supplementation with nutrient materials (Casas Lopez et al. 2004; Rodriguez Porcel et al. 2007; Bizukojc et al. 2012). In date syrup, glucose was first consumed and fructose assimilation due to catabolic repression of glucose was ceased (Hajjaj et al. 2001). During glucose and fructose utilization, no cessation of lovastatin production was observed due to substrate shifting because nearly the same metabolism is involved in glucose and fructose consumption. Study in bioreactor was ceased after 12 days when lovastatin biosynthesized was being at high titer, because in 12 days the biomass production was very high and agitation rotor cannot work due to viscosity of environment, therefore we had to stop the experiment.

As observed, *A. terreus* shifted to fructose consumption before glucose was fully exhausted. Junior et al. reported that a simultaneous consumption of glucose and fructose by *Saccharomyces cerevisiae* is strongly affected by the structure complexity of the nitrogen source (Júnior et al. 2008). The optimized date syrup-based medium has a complex nitrogen source; it was perhaps the main reason for the mechanism of sugar assimilation by the fungi, namely the observed diauxic growth.

Overall glucose productivity expressed by yield coefficient (Y_{xp}), (9.69 mg/g DW), was better than that of fructose, (3.45 mg/g DW). The yields of lovastatin production appeared higher during growth on glucose, if compared to fructose. Overall Y_{sp} were 5.8 and 3.57 mg/g for glucose and fructose respectively.

Analysis of biomass on substrate yields (Y_{sx}) also showed that fructose was more suitable for biomass production than glucose, 0.59 and 1.03 gDW/g respectively. These yields were higher than those reported in the literature 0.18 gDW/g according to Jia et al. (2009b). The reason of amount of biomass on substrate yields was more than 1 when assimilation in fructose in our study is the existence and assimilation of some little glucose in the medium.

Average specific biomass growth rates for glucose and fructose were 0.102 and 0.195 h^{-1} respectively. This value in fructose was significantly higher than the values reported by other authors, 0.03 h^{-1} using fructose, lactose, and glycerol (Lopez et al. 2003), 0.025 h^{-1} using lactose and about 0.005 h^{-1} with glucose (Lai et al. 2007), 0.168 h^{-1} in medium containing glucose and lactose (Hajjaj et al. 2001), 0.172 h^{-1} for sucrose, 0.15 h^{-1} for glycerol, and 0.13 h^{-1} for soluble starch (Jia et al. 2009b). This study shows that growth of *Aspergillus terreus* in date syrup was amazingly fast. It could be due to the presence of carbohydrates like fructose and glucose and mineral elements like Zn and Fe in suitable concentration for lovastatin biosynthesis and biomass production (Szakács et al. 1998; Kumar et al. 2000; Hajjaj et al. 2001; Lai et al. 2002; Casas Lopez et al. 2004; Bizukojc et al. 2007; Lu et al. 2010).

Conclusion

According to the production amount of date in the world and Iran, date syrup can be assumed as an available and cheap carbohydrate source for microbial cultivation. To our knowledge, the use of date syrup for lovastatin production has been studied for the first time. This study demonstrated that date syrup, an agricultural product produced from low-quality date palm, supplemented with a nitrogen source, is the appropriate medium for lovastatin production by *Aspergillus terreus* ATCC 20542 in submerged cultivation and can be a suitable candidate for industrial application. Upon RSM experiment, the optimal lovastatin production was observed for concentration of date syrup carbohydrates equal to 64 g/l, yeast extract 15 g/l, pH 6.5, and agitation speed 150 rpm. Data show that pH has an adverse effect on lovastatin biosynthesis and biomass production, high pH suitable for biomass formation, and low pH suitable for lovastatin biosynthesis, although the role of pH on lovastatin biosynthesis in date syrup medium is very weak. The batch culture in these optimal conditions was performed in a stirred tank bioreactor and after 12 days, 241.1 mg/l lovastatin was biosynthesized and biomass concentration reached (43 gDW/l). This result showed that date syrup is an excellent medium for growth of *Aspergillus terreus* due to being rich with carbohydrates and trace elements what is favorable for *A. terreus*. Nevertheless, the presence of glucose and fructose caused the diauxic growth of *A. terreus*. Kinetic and yield parameters revealed that glucose is a more suitable carbohydrate source for lovastatin biosynthesis and fructose increases biomass production. We recommended that in order to obtain more biosynthesis of lovastatin, use fed-batch strategy because this strategy dilute viscosity of medium which emerge due to high growth of *Aspergillus terreus* and therefore more lovastatin biosynthesis will happen in stationary phase in stirred tank bioreactor.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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