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Full Length Research Paper

Standardization of growth and fermentation criteria of *Lasiodiplodia theobromae* for production of jasmonic acid

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Aim of the study was to examine the enhanced growth supporting conditions and production of jasmonic acid (JA) by *Lasiodiplodia theobromae* in liquid cultures. Seven different media were assessed for supporting the maximum growth of the *L. theobromae*. Basal salt media supported maximum growth on solid as well as liquid media. Optimum temperature and pH for growth of *L. theobromae* were found 30°C and 5.5, respectively. Besides studying influence of these environmental factors for growth, some other basic fermentation criteria for improvement of fermentation of JA using *L. theobromae* were standardized. This includes inoculum size and age, effect of aeration, effect of surface area and effect of sub-culturing the fungus.

Key words: Jasmonic acid, high performance thin layer chromatography, *Lasiodiplodia theobromae*, submerged fermentation.

INTRODUCTION

Industrial bioprocesses with filamentous fungi embrace the production of majority of commercially important products of biotechnology, in the sense of quantity as well as the diversity of metabolites. These are mainly the submerged culture processes, where a dynamic relationship exists between environmental conditions and the growth pattern of these modular microorganisms (Žnidaršič and Pavko, 2001). Jasmonic acid (JA) [3-oxo-2-(2'-cis-pentenyl) cyclopantane-1-acetate] fermentation is of importance in perfumery industries. The methyl ester form of JA, methyl JA, was first isolated from the essential oil of jasmine (*Jasminium grandiflorum*) in 1662 by Demol et al. (reviewed in Ueda and Miyamoto, 1998) and from Tunisial rosemary (*Rosmarinus officinalis* L) in 1967 (reviewed in Ueda and Miyamoto, 1998) as an odoriferous compound. The structure of this compound was elucidated but no biological activity was found at that time. In 1971, JA was first isolated from the culture filtrate of *Lasiodiplodia theobromae* (the synonym of *Botryodiplodia theobromae*) and its role was found as a plant growth regulator (Alderidge et al., 1971). JA has also been found to play role in signaling for plant defense system (Staswick and Tiryaki, 2004; Mita et al., 2005).

Methyl jasmonate, a methyl ester of JA is a pleasant flavoring compound. Currently it is extracted from the flowers of *Jasminium grandiflorum* and used in the manufacturing of high-grade perfumes. A large number of flowers are needed to produce a small amount of essential oil. It takes 1000 pounds of petals to make approximately two pounds of rose oil. This is equal to 30 roses to make one drop of essential oil. This is a very expensive and time-consuming process that accounts for the high price of these oils or absolutes. To avoid the higher prices of true, natural and pure absolute oil, trading companies often offer absolute oils that are diluted with up to 90% vegetable oil. This does not affect or damage the healing properties and the scent is still strong since these oils are highly concentrated. In fact, it is recommended to use them diluted. (http://www.nature-saroma.com/info/eoproduction.html, 2006). Estimated global demand for jasmine concrete is 500 tonnes / annum. In India price of jasmone concrete is about 15000 Rs/kg with 60-tonnes/annum demands, which is likely to increase at the rate of 7-8% per annum.

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Our aim is to produce JA by fermentation of *L. theobromae* in a cost effective manner. In this study we have studied influence of environmental factors, such as media, temperature and pH, for growth of *L. theobromae*. Also, we have standardized basic fermentation criteria for higher production of JA by fermentation of *L. theobromae.*

MATERIALS AND METHODS

Microorganism

L. theobromae (MTCC 3068) was obtained from Microbial Type Culture Collection (MTCC) Institute of Microbial Technology, Chandigarh, India. This strain was maintained on potato dextrose agar (Hi-Media Mumbai, India). Slants were incubated at 28°C for 3–4 days and later stored at 4°C and sub-cultured every month.

Chemicals and solvents

(±) JA was purchased from Sigma, Bangalore, India and used as a standard reference compound for quantification of JA. All dehydrated media purchased from Hi-media, Mumbai, India. All solvents were purchased from Qualigens, Mumbai, India.

Cultural technique

Sample of stock culture was transferred to potato dextrose agar plates and incubated for 3 days at 30°C. Agar plug (8 mm dia) was cut with sterile cork borer and used for inoculation.

Selection of media and physical conditions for maximum growth of *L. theobromae*

Seven different media; two chemically defined (Basal salt medium composition g/l: sucrose, 5; $NaNO_3$, 7.5; KH_2PO_4, 2.0; KCl, 0.3; $MgSO_4$. $7H_2O$, 0.6; $FeSO_4$. $7H_2O$, 0.6; $ZnSO_4$. $7H_2O$, 0.03; $MnSO_4$. $7H_2O$, 0.003; $CuSO_4$. $7H_2O$, 0.003; Na_2MoO_4. $2H_2O$, 0.003; yeast extract, 1.0; pH 5.5 (Eng et al, 1998) and czapek Dox medium) five complex media (Corn Meal media pH 6.2±0.2, Malt Extract media pH 6.0±0.2, Nutrient Agar pH 7.3±0.2, Potato Dextrose media pH 5.6±0.2 and Wort media pH 4.8±0.2) were selected for screening of maximal growth supporting media. All the media were prepared, sterilized at 121°C for 15 min, than plated and pre-incubated for 24 h. Inoculation was done by placing three day old grown *L. theobromae* agar plug in the center of the plate and incubated at 30°C. Colony diameter was recorded at various time intervals and from that colony spreading rate was calculated. Maximum growth supporting media were further compared amongst themselves by growing fungus in liquid media. Inoculated broths were incubated at 30°C and after 8 days growth was measured as dry mycelial weight (DMW). Maximum growth supporting media was used for further studies. 100 ml of the selected media in 250 ml Erlenmeyer flasks was inoculated and incubated at different temperatures (25, 27, 30, 32 and 36°C) for 8 days. At the end of incubation period optimum temperature for growth was determined from DMW measurement. Optimization of the initial pH of the media was determined in the range of pH 4-8 with each 0.5 unit increment. Final pH and DMW was measured on the 8th day.

Factors affecting JA production

Optimization of inoculum size: Different number of agar plugs (1, 2, 4, and 8 agar plugs) were cut from two-day old grown fungus (log phase) and inoculated in to the maximum growth supporting media and incubated at optimized physical conditions for 8 days. After 8 days growth and JA production was measured.

Effect of substrate concentration: Since at the time of maximum growth (8 day) and max JA production, available substrate (sucrose) was depleted from the broth, we ran another fermentation cycle by doubling the substrate concentration to confirm whether JA production is limited by substrate availability or not. All other parameters of the fermentation were kept same.

Effect of agitation: Changes in growth and JA production in the agitation conditions were studied. Flasks inoculated were kept on shaker at 125 rpm and samples were withdrawn on everyday 3rd day onward till 8th day at 30°C. Biomass production, substrate consumption, broth volume, change in pH, and JA production were assessed from the sample withdrawn.

Batch fermentation: With all the physical condition standardized, batch fermentation cycle was carried out in static condition for 8 days, sample were withdrawn each day and analyzed for change in volume of broth, change in pH, utilization of carbohydrate, biomass production and JA production**.**

Influence of surface area: Influence of surface area on JA was studied by using the vessels of various sizes. Erlenmeyer flasks of 250, 500 and 1000 ml capacity containing 100 ml of BSB (pH 5.5) were inoculated with single agar plug and incubated at 30°C for 8 days. Change in pH, JA produced and biomass formed were analyzed after 8 days.

Effect of sub-culturing: Various sub-cultured (that is after 8, 15 and 17 transfer) and fresh strain of *L. theobromae* were analyzed for JA producing ability after 8 days to study effect of sub-culturing on JA production.

Analytical methods

Substrate utilization: Substrate consumption was determined by phenol sulphuric acid method (Dubois et al., 1956 cited in Thimmaiah, 1999). The concentration of sucrose was determined as total sugar present as hexose.

Growth determination: Biomass production was determined by dry weight after broth filtered on pre-weighted Whatman No.1 filter paper followed by drying at 60°C for 24 h.

Extraction and measurement of JA: JA was extracted from fungal cultural filtrate of L. theobromae after acidification to pH 2-3 with 6 M HCl and extracted with equal volume of ethyl acetate. Extract was concentrated to 100 times. JA measurement was carried out with high performance thin layer chromatography (HPTLC). Concentrated extract was loaded on silica gel 60 F_{254} aluminum foils (Merck, Germany) along with standard JA using Linomate-5 spray on applicator (Camag, Switzerland) of HPTLC under the flow of N_2. Foils were ran with iso-propanol**:** ammonia: water (10: 1: 1 v/v) (Ueda and Miyamoto, 1994). After running of foils they were scanned with scanner-3 (Camag, Switzerland) and quantified with the help of winCATs software ver. 1.2.2 by measuring density of the JA band separated on the TLC foils.

RESULTS AND DISCUSSION

Media selection

Amongst the seven different growth supporting media tested for growth of *L. theobromae*, basal salt media was supporting the highest growth followed by wort agar and potato dextrose agar (Figure 1). Colony spreading rate was also found higher with the basal salt agar followed by the wort agar and potato dextrose agar. Basal salt media, wort agar and potato dextrose agar were further tested

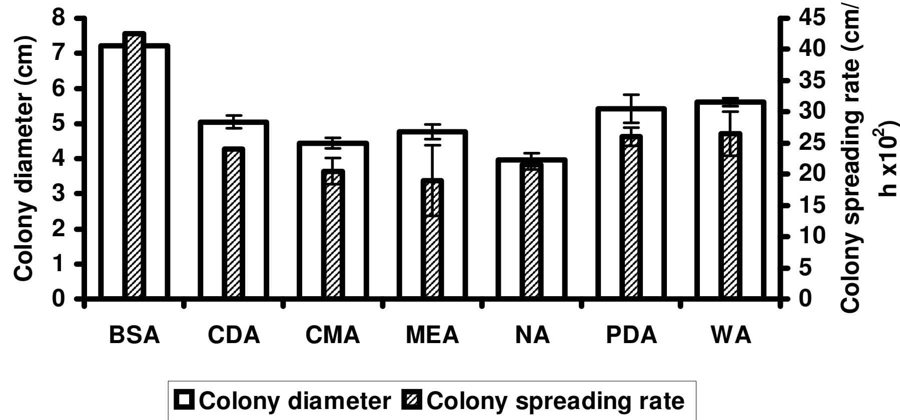

Figure 1. Growth comparison of *L. theobromae* in various media. BSA, basal salt agar; CDA, Czapex dox agar; CMA, corn meal agar; MEA, malt extract agar; NA, nutrient agar; PDA, potato dextrose agar; WA, wort agar along with colony spreading rate.

for their ability to support the growth in liquid culture. It was found that the basal salt media remained the best media for the better growth however; the potato dextrose broth supports more growth of *L. theobromae* in the liquid state in comparison to wort broth (Figure 2).

Temperature optimization

Temperature is an important factor for growth and secondary metabolites production by microorganisms. Maximum growth was obtained at 30-32°C (Figure 3). However, the maximum temperature for growth does not always correspond to the maximum secondary metabolite production. From the literature it was found that for *Lasiodiplodia,* temperature for maximum biomass and JA production remains same. Eng et al. (1998) reported that maximum biomass for *Botryodiplodia theobromae* was obtained at 30°C and maximum production was also obtained at same temperature. These results also agree with the previous studies that JA production by *B. theobromae D7/2* was maximum at 27-30°C (Gűnther et al., 1990).

Effect of initial pH

Effect of initial pH of medium on growth of *L. theobromae* was assessed in the range 4.0 to 8.0 with each successive 0.5 unit increment. Figure 4 represent the change in final pH and biomass produced in basal salt medium at different pH after 8 days of incubation at 30°C. Maximum biomass produced was 10.96±1.17 g/l when initial pH was 5.5. Eng et al. (2003) reported that initial pH of the culture media did not affect growth density and radial growth. However we found that initial pH of the medium was affecting the biomass produced by the culture of *L. theobromae*.

Effect of inoculum size

Increase in inoculum size from one mycelial-agar plug to

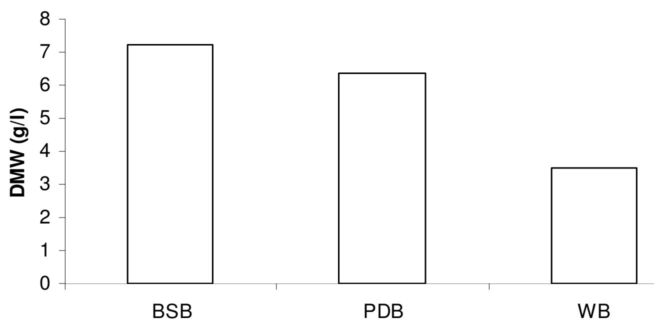

Figure 2. Dry mycelia weight in the liquid culture (BSB- Basal salt broth; PDB- Potato dextrose broth; WB- Wort broth).

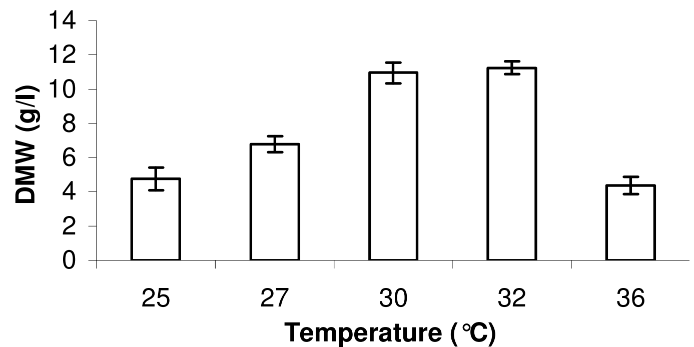

Figure 3. Effect of temperature on dry mycelia weight of *L. theobromae* on 8th day.

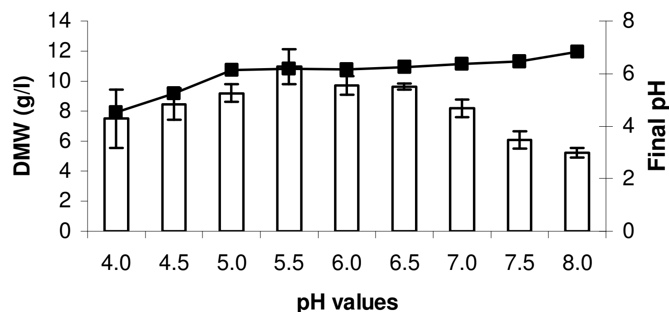

Figure 4. Effect of initial pH on mycelial dry weight and final pH (■) of the broth of *L. theobromae*.

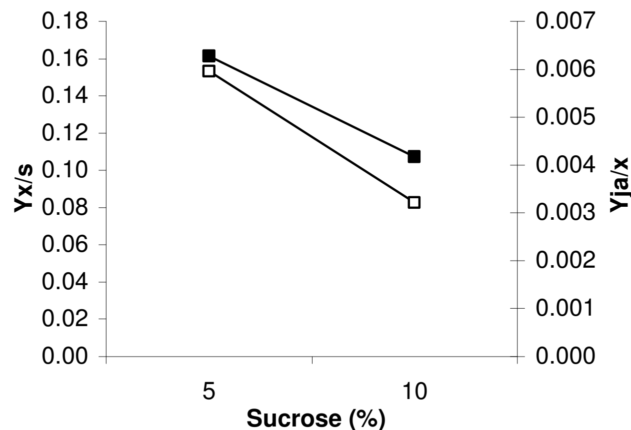

Figure 5. Effect of the increase in sugar concentration on Yx/s (■) and Yja/x (□) in jasmonic acid production during the growth of *L. theobromae* static culture.

eight mycelial-agar plugs did not show significant difference in the yield of JA, although the growth was slightly increased as the inoculum size was increased. So inoculation with single mycelial-agar plug was sufficient for higher yield of JA (Table 1).

Effect of substrate concentration

To confirm that growth is not limited due to lack of substrate concentration, the effect of increase in substrate i.e. sucrose on biomass (Yx/s, g biomass/g sucrose) and yield of JA (Yja/x, g JA produced/ g biomass) was studied (Figure 5). Yx/s was found 0.161 g/g at 5% sucrose. However, it decreased to 0.107 g/g on doubling the sucrose concentration. Reduction in the Yx/s at higher sucrose concentration might be related to the production of an extracellular polysaccharide. Increase in viscosity at higher sucrose concentration might also affect the growth. Yja/x was also decreased with the increase in sucrose concentration in the similar manner that observed with Yx/s. Cortezi et al. (2005) reported that an increase in sugar concentration can inhibit the dextranase production due to high viscosity of medium which decrease cellular growth.

Table 1. Effect of inoculation size on dry mycelial weight (DMW) and JA production on 8th day.

No. of mycelial-agar plug	DMW (g/l)	JA (mg/l)	Yja/x (g/g)
1	10.73	56.12	0.005230
2	10.82	54.62	0.005048
4	10.56	53.21	0.005039
8	11.02	57.3	0.005200

Effect of agitation

In agitation and static condition, maximum biomass was obtained on 3rd and 6th day, respectively. However, Maximum JA produced in agitation condition was 22.51 mg/l on the 3rd day whereas in static condition it produced 59.94 mg/l of JA on 7th day (Figure 6). Our results are in corroboration with the previous studies; Eng et al (1998)

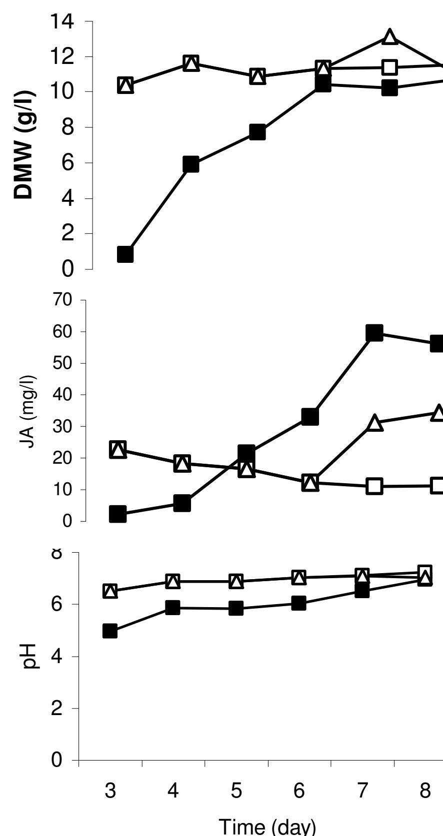

Figure 6. Kinetics of cell growth (a), jasmonic acid production (b) and pH (c) by *L. theobromae* grown in static condition (■), agitation at 125 rpm (□) and agitation for first 5 days and than kept on static condition (Δ).

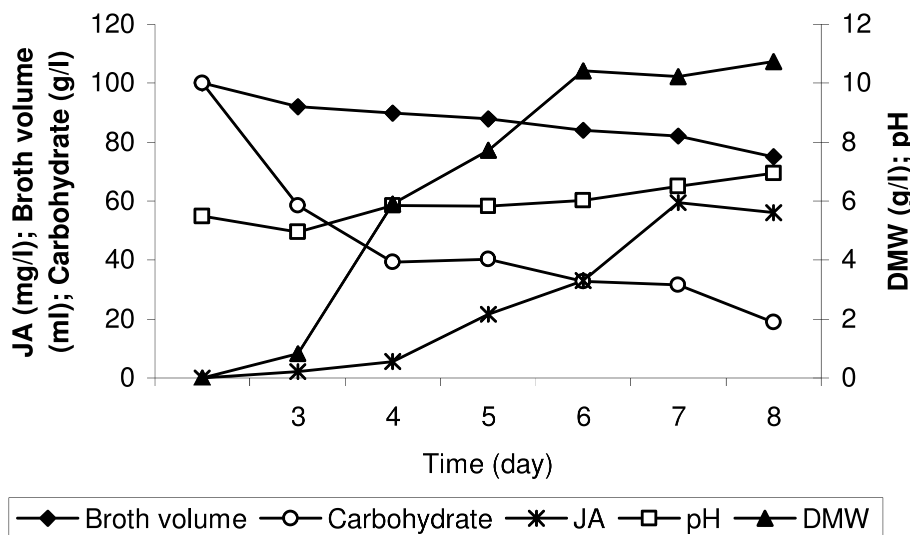

Figure 7. Fermentation profile of *Lasiodiplodia theobromae* for the production of jasmonic acid.

reported that JA production was higher in the static condition than agitation. The growth pattern observed in both the conditions was different. In agitation *L. theobromae* formed pellets and in static condition it formed mycelial mat on surface of the broth, indicating that this fungi is aerobic. Morphological characteristics of mycelial-submerged cultures have been established as one of the key bioprocess parameters (Žnidaršič and Pavko, 2001). We have also studied the effect of dual conditions- agitation and static- on the biomass and JA production. The culture was kept in shaking condition for five days and than transferred to the static condition. It was found that when the culture was transferred after initial agitation period to the static condition, production of JA increased to more than double compared to agitation condition, however it was less than JA produced in static condition. So we conclude that JA production is favored in static condition. During the course of fermentation, increase in the pH was observed.

Batch fermentation

A typical fermentation profile was seen in the production of JA using *L. theobromae* in static conditions (Figure 7). Culture entered in to log phase after 3rd day of growth and continued till 6th day. JA was detected in the cultural broth after 3rd day and reached maximum value of 59.96 mg/l on 7th day and thereafter declined to 56.12 mg/l on 8th day with associated change in pH from 6.51 on 7th day to 6.95 on 8th day. Thereafter reduction in JA concentration was observed. The reduction of JA after 7th day might be related to consumption of JA due to carbon limitation or change in pH in the cultural medium. However, JA production was related to change in pH of the broth. At pH values higher than 6.0, JA concentration was considerably higher so for enhanced growth optimum pH is 5.5 whereas for higher JA production, pH required more than 6.0. Infect we found that on 2nd day of growth of *L. theobromae* reduces the pH and then the pH was slowly raised to 6.0 by 6th day. Increase in the pH of fermentation broth could be due to depletion of nitrate ion by *L. theobromae* in cultural medium, which generates alkaline pH by ionization of sodium ion. *B. theobromae* biosynthesizes only (+)-7-iso-JA isomer (Alderidge et al., 1971). Side chains of *cis*-(+)-7-iso-JA are oriented being more unstable than *trans*-oriented isomer. Koda (1992) reported that at pH values higher than 7 the *cis* (+)-7-iso-JA isomerizes to the *trans* (-)-JA isomer, resulting in equilibrium of an about 95:5 *trans* : *cis* ratio. It could be depicted that JA was synthesized in the early stationary phase as the maximum growth was observed on 6th day and maximum JA produced on 7th day, a typical characteristic of secondary metabolite.

Influence of surface area

In static condition *L. theobromae* was forming a mat on the surface of the medium therefore it was important to study the effect of available surface area on the JA production. Effect of surface area was studied measuring the yield of JA by increasing the vessel size. Increase in surface area lead to increase in JA production (Table 2). Almost two-fold and two and half-fold increase in the JA production was observed when vessel size was increased from 250 ml capacity flask to 500 and 1000 ml capacity flask respectively. Biomass produced did not increase with the increase in the vessel size. 500 ml capacity Erlenmeyer flask with the 100 ml of medium produced

Table 2. Influence of surface area on jasmonic acid production.

Vessel size (ml)	Remaining broth volume (ml)	Final pH	DMW (g/ l)	JA produced (mg/l)	Yield p/x (mg JA/ g biomass)
250	72	6.8	9.34	73.66	7.88
500	64	7.22	12.67	150.56	11.88
1000	55	8.33	9.99	171.25	17.14

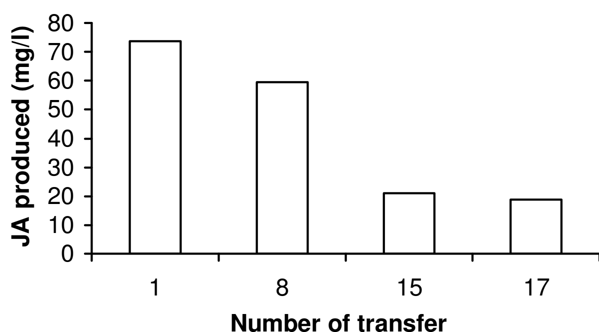

Figure 8. Influence of repeated transfer on jasmonic acid production ability of *L. theobromae*.

higher biomass probably due to availability of higher surface area whereas due to available surface constrain in 250 ml flask and probably due to less physical support from the base in 1000 ml flask, less biomass was produced. Overall increase in JA productivity and final pH was observed with the increase in vessel size.

Effect of sub-culturing

For any fermentation process it is of prime importance to maintain the higher productive strain. After each transfer to the fresh medium, at each cell division, there is small probability of mutation occurring and repeated subculturing involves very much such divisions and a high probability of strain degeneration (Stanbury et al., 1997). When we compared JA produced by fresh and variously sub-cultured (after 8, 15 and 17 transfer) strain, it was found that sub-culturing resulted in reduction of JA production ability of *L. theobromae* (MTCC-3068) (Figure 8).

CONCLUSION

Highest growth of *L. theobromae* was observed in basal salt media at initial pH 5.5 at 30°-32°C. Biomass increased rapidly in agitated culture media whereas JA production was enhanced in static condition. Maintenance of pH near 6.5 or more increased the JA production. The surface area of the medium also influenced JA production. Fermentation of *L. theobromae* in completely controlled bioreactors with these standardized cultural conditions will give high yield of JA.

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REFERENCES

Alderidge DC, Galt S, Giles D, Turner WB (1971). Metabolites of *Lasiodiplodia theobromae*. J. Chem. Soc. pp. 1623-1627.

Broadbent D, Hemming H and Trner W (1968). Preparation of Jasmonic acid, GB paent 1286266, C07 C61/36.

Cortezi M, Monti R, Contiero J (2005). Temperature effect on dextransucrase production by *Leuconostoc mesenteroides* FT 045 B isolated from Alcohol and Sugar Mill Plant. Afr. J. Biotechnol. 4(3): 279-285

Creelman RA, Mullet JE (1995). Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc. Natl. Acad. Sci. (USA) 92: 4114-4119.

Eng F, Gutierrez-Rojas M, Favela-Torres E (1998). Cultural conditions for jasmonic acid and biomass production by *Botryodiplodia theobromae* in submerged fermentation. Process Biochem. 33(7): 715-720.

Eng F, Gutierrez-Rojas M, Favla-Torres E (2003). A survey of temperature and pH effect on colonial growth of *Botryodiplodia theobromae* RC1. Rev. Iberoam Micol. 20: 172-175.

Farbood MI, Brlocker RW, McLean LB, Sprecker MA, McLean MP, Kossiakoff N, Kim AY, Hagedron M (2002). United States Patent. No. 6,458,569.

Gundalch H, Mueller MJ, Kutchan TM, Zeick MM (1992). Jasmonic acid is a signal transducer in elicitor induced plant cell culture. Proc. Natl, Acad. Sci. USA. 89:2389.

Günther T, Miersch O, Wolfgang F, Sembdner G (1990). Synthetic medium for manufacture of 7-iso-jasmonic acid with *Botryodiplodia theobromae*, Patent DD. WP 279688 A1 12 P7/40.

Koda Y (1992). The role of jasmonic acid and related compounds in the regulation of plant development. Int. Rev. Cytol. 135: 155-159.

Mita G, Quarta A, Fasano P, Paolis AD, Sansebastiano GPD, Perrotta C, Iannacone R, Belfield E, Hughes R, Tsesmetzis N, Casey R, Santino A (2005). Molecular cloning and characterization of an almond 9-hydroperoxide lyase, a new CYP74 targeted to lipid bodies. J. Exp. Botany, 419(56): 2321–2333.

Mueller MJ, Brodschelm W (1994). Quantification of jasmonic acid by capillary gas chromatography-negative chemical ionization-mass spectrometry. Anal. Biochem. 218: 425-435.

Stanbury PF, Whitaker A, Hall SJ (1997). The isolation, preservation and improvement of industrially important microorganisms, In "Principles of fermentation technology" 2nd Eds. pp 35-85.

Staswick PE, Tiryaki I (2004). The Oxylipin Signal Jasmonic Acid Is Activated by an Enzyme That Conjugates It to Isoleucine in Arabidopsis. The Plant Cell, 16: 2117–2127.

Thimmaiah SK (1999). Carbohydrates, in "Standard methods for biochemical analysis" Kalyani publishers, New Delhi, India. pp 55-57.

Ueda J, Miyamoto K (1994). Separation of a new type of plant growth regulator, jasmonates, by chromatograohic procedures. J. Chromatogr. A, 658: 29-142.

Žnidaršič P, Pavko A (2001) Morphology of Filamentous Fungi. Food Technol. biotechnol. *39* (3): 237–252.