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Comparative study of GABA production from *Monascus purpureus* and its co-culture with *Monascus sanguineus.*

Rashmi Dikshit, and Padmavathi Tallapragada*.

Department of Microbiology, Centre for PG Studies, Jain University, 18/3, 9th Main road, 3rd Block, Jayanagar, Bangalore – 560011, Karnataka India.

ABSTRACT

The aim of present study was to explore and compare the γ-aminobutyric acid (GABA) production from *M. purpureus* and co-culture of *M. purpureus* and *M. sanguineus* under solid state fermentation. Different agro wastes were screened and coconut oil cake was found to be the best substrate among them. Production of GABA as secondary metabolite was confirmed through thin layer chromatography (TLC) and its concentration was estimated by means of simple ninhydrin protocol. Plackett-Burman design was used to screen physical and nutritional parameters and to compare the GABA yield from *M. purpureus* and co-culture of *M. purpureus* and *M. sanguineus*. The production of GABA from co-culture of *M. purpureus* with *M. sanguineus* was found inhibited as compared to its mono culture. An average reduction of approximately 17% was observed with co-culture.

Keywords: *M. purpureus*; co-culture; ninhydrin protocol; GABA

Corresponding author

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INTRODUCTION

GABA is a non-protein amino acid, known to have several important roles in inhibitory neuro-transmitter in mammalian nervous system. It is also known to contain other physiological applications such as regulation of hormones secretions, hypotensive effect, management of tranquilising excitement, epilepsy therapy, improving memory, etc. GABA also plays an important role in enhancing the functional activity of kidney and liver [1]. GABA can prevent diabetes while acting as a strong secretagogue for insulin from the pancrease [2]. Regular intake of GABA can prevent anxiety, promote inhibition in cancer cell proliferation and regulate lipid serum levels [3]. GABA therefore can be classified as a bioactive compound in foods and pharmaceuticals [4].

Due to above beneficial properties, recently enhancement in GABA production has taken importance in research by various means. The demand of GABA enriched food also has been increased and several attempts have been made to develop a functional food containing good amount of GABA content. Few of these include fermented food products made from lactic acid bacteria such as cheese [5], Tempeh-like fermented soybean, Korean kimchi and yogurt [6] etc. In nature, GABA is also widely distributed in various forms such as in sprouted-beans, soybean seedling, tea leaves, grain germs and microorganisms etc. [7]. Several microorganisms viz. *Monascus, Rhizopus, Lactobacillus paracasei, Lactobacillus brevis, Lactococcus lactis, Streptococcus thermophilus* etc have been identified and exploited for GABA production [8]. *Monascus* strains are extensively used to produce red pigment, which contains different health beneficial secondary metabolites. Traditionally, three strains, i.e. *Monascus purpureus*, *Monascus ruber* and *Monascus pilosus* have been used to produce different secondary metabolites along with red pigment under fermentation condition in food industry for centuries in East Asia [9]. GABA production through biosynthesis approach can be more promising than synthetic way since it has a high catalytic efficiency, simple reaction procedure and environmental compatibility [10]. It engrosses bioconversion of decarboxylating glutamate to GABA and reaction catalyzed by glutamate decarboxylase (GAD). For cultivation and production of GABA from microbial sources , till now assorted techniques such as immobilized cell technology, sourdough fermentation, batch fermentation method have been exploited [11]. Certain physical factors such as temperature, pH, inoculum size etc. and chemical composition as media additives in fermented substrates are considered to be important factors for enhancing the GABA yield.

The aim of present study was to explore and compare the GABA production from *M. purpureus* and co-culture of *M. purpureus* and *M. sanguineus* under solid state fermentation. Different agro wastes were screened and coconut oil cake was found best substrate among them. Plackett-Burman design was used to screen physical and nutritional parameters and compare the GABA yield from *M. purpureus* and Co-culture of *M. purpureus*. TLC was done to confirm the presence of GABA production. Concentration of GABA content in the sample was estimated with known ninhydrin method.

MATERIAL AND METHODS

Culture

Monascus sanguineus was isolated from spoiled pomegranate and reference strain *M. purpureus* MTCC 410 procured from MTCC Chandigarh India. Potato Dextrose Agar (PDA) media was used to maintain the both strains and incubated at room temperature (30°C) for 10 days [12].

Inoculum preparation

The spore suspension was prepared separately for each strain in 0.9 % saline water under aseptic condition. Spore concentration was maintained approximately 5×10^7 spores/ml for both mono and dual inoculation. For co-culture, these seed cultures were mixed in the ratio of 1:1[13].

Solid-state fermentation for GABA production

Solid substrates viz. wheat bran, tamarind seed, coconut oil cake and jack fruit seed were used for GABA production from *M. purpureus* and co-culture of *M. purpureus* and *M. sanguineus.* Five g of each substrate was placed in a 250 ml conical flask separately and 30 ml of basal media was added. For basal media

composition, 100 g dextrose, 10 g peptone, 2 g KNO_3, 2 g NH_4 H_2 PO_4, 0.5 g $MgSO_4.7H_2O$, and 0.1 g $CaCl_2.2H_2O$ were dissolved in 1,000 ml distilled water. The pH of the medium was adjusted to pH 6.0 [14].

TLC for confirmation of GABA

GABA presence from *Monascus purpureus, M. sanguineus a*nd co-culture extracts were done by TLC. For this, 10 µl extract from all samples along with GABA standard was spotted on silica gel 60F254 Aluminium sheets (Merck, Germany). Used mobile phase was n-butanol: Acetic acid: water (5:3:2,v/v) [15].

Extraction and Calorimetric estimation of GABA

Zhang and Bown [16] protocol was used for GABA extraction. Calorimetric estimation of GABA concentration was done according to Dikshit and Tallapragada [14]. Ninhydrin protocol was used for estimation of GABA concentration in the sample and absorbance was measured at 570 nm against a blank prepared simultaneously without ninhydrin solution. Different concentrations of standard GABA were prepared and estimated through above said protocol.

Plackett-Burman design for screening of parameters

To examine physical and nutritional parameters for GABA synthesis in our study, Plackett-Burman design was attempted. Since, coconut oil cake was identified as the most appropriate substrate for GABA production; it was used as a substrate for screening other parameters for better yield. Seven factors viz. inoculum size, incubation period and pH as physical parameters and malt extract, dextrose, ammonium sulphate and monosodium glutamate as nutritional parameters were screened. All parameters were explored in two levels (Table 1), high (positive) and low (negative).

Plackett–Burman designs are the experimental designs that are used for examining the dependency of any measured output on a number of independent variables using a limited number of experiments to minimize the variance of the estimates of these dependencies. Interactions between the factors were considered negligible. In other words, this technique allows the assessment of X number of variables in X + 1 number of experiments. The constraint being that it assumes that there are no interactions between the variables [17]. Hence the Plackett-Burman design was applied for the screening of seven parameters and as per the design philosophy, only 8 runs were carried out. As per the limitation of the design, the interaction effects of the media components were disregarded and only the linear effects were taken into account. The Plackett-Burman design for our experiment can be represented by the following polynomial equation:

$$GABA\ yield\ \left(\frac{mg}{gds}\right) = A_0 + \sum_{i=1}^{n} B_i x_i \dots\dots\dots\dots\dots\dots\dots (1)$$

Where A_0 is the model co-efficient, B_i, the coefficients of the linear variables, x_i, the variables and n, the number of media components for the study (7 in this case). Each of these seven media components were represented in two levels, one high and one low (indicated by positive and negative sign respectively in Table 1).

The response value effect (RVE) of each of the media components was calculated by the following equation:

$$\text{RVE of tested variable } E_{x_i} = \frac{\sum M_{i_+} - \sum M_{i_-}}{N} \dots\dots (2)$$

Where the first and second terms of the numerator denote the summation of the response values at positive (high) level and negative (low) level respectively and N is the number of runs carried out for the experiment.

Statistical Analysis

Regression and graphical analysis of the experimented data was done with the help of MATLAB® software Version 7.5.0.342 (R2007b) from The Math Works, Inc. ANOVA was used to estimate the statistical parameters (p-value < 0.005).

RESULTS AND DISCUSSION

Substrates for GABA production

Utilizing synthetic media for the production of bioactive compounds from microbial sources at industrial scale is not economical. Keeping this in mind, different agro-waste residues as solid substrates were screened for GABA production. Among them, coconut oil cake was found appropriate for both *M. purpureus* as well as co-culture. The GABA yield for *M. purpureus* was observed as 6.3 mg/gds followed by jack fruit seed (3.4 mg/gds) whereas with wheat bran and tamarind seeds the yield was not much appreciable (Fig.1). Generally coconut oil cakes are known to be rich in fibre, protein and energy contents. Hence, it is one of the most promising substrates in developing bioprocesses for the production of bio-molecules and other secondary metabolites from microbial sources. It is also a good alternative source for solid state fermentation on traditional applications for generation of eco friendly bio fuel. Another major factor that favours utilisation of oil cakes for bioprocess is their low cost. Its' low cost accessibility throughout the year makes it even more favourable when it comes to economical viability [18].

TLC for GABA confirmation

GABA production from both *Monascus purpureus* and co-culture was confirmed with TLC. All fermented substrates along with GABA standard had given the same retention factor (0.56) (fig. 2).

Examination of GABA yield utilizing the Plackett-Burman design

Physical as well as nutritional parameters were examined to find out significant factors for GABA production for both *M. purpureus* and co-culture. Significant variables for GABA yield were investigated with the help of t-value. Obtained data for GABA yield revealed that incubation period, MSG and pH had positive effect whereas the rest of the variables had negative effect for *M. purpureus* as well as co-culture (Fig.3).

Similarly the response plots were analyzed for the linear effect of tested variables in order to compare the optimized yield for both *M. purpureus* and co-culture (Fig. 4). These response plots help in understanding the linear effect of one variable with respect to other, while keeping remaining variables at one fixed value. The trend for GABA yield with inoculum size and incubation period as parameters were similar for both *M. purpureus* and the co-culture with the yield declining with increase in the size of inoculums or the duration of the incubation (Subplot 1 of Fig. 4). The highest GABA yield for co-culture was noticed as 6.9 mg/gds with an incubation period of 9 days and minimum tested inoculum size of 5 % v/v. The yield decreased as either or both of these parameters were increased. *M. purpureus* too showed similar behaviour yielding maximum GABA of 8.1 mg/gds at 9 days of incubation and 5 % v/v inoculums size. The minimum GABA yield for *M. purpureus* was 5.8 mg/gds. Hence, the GABA yield obtained by *M. purpureus* had an average positive offset of about 1.4 mg/gds as compared to the co-culture for the entire range of the parameters under study.

The GABA yield for co-culture with incubation period and pH as variables also showed similar tendency yielding maximum GABA of 6.5 mg/gds with an incubation period of 9 days and inoculum size of 5 % v/v (Subplot 2 of Fig. 4). For *M. purpureus*, the maximum and minimum GABA yield was noticed as 7.76 mg/gds and 6.16 mg/gds respectively. The GABA yield for both *M. purpureus* and co-culture decreased as the pH increased from acidic towards basic. The yield also declined as the duration of the incubation increased, however the rate of reduction was comparatively steeper in case of co-culture. In this case also, an average positive offset in GABA yield of about 1.4 mg/gds was observed with *M. purpureus* as compared to the co-culture.

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The GABA yield for *M. purpureus* and co-culture with inoculum size and pH as variables too showed a positive offset of approximately 1.35 mg/gds for *M. purpureus* (Subplot 3 of Fig. 4). However the rate of decrease of GABA yield for either *M. purpureus* or the co-culture was almost similar with these variables.

The results for GABA yield for *M. purpureus* and co-culture with monosodium glutamate and dextrose as variables showed a distinctive trend (Subplot 4 of Fig. 4). The GABA yield for *M. purpureus* showed an increasing trend with dextrose concentration contrary to the co-culture which showed a decreasing trend. However at high MSG concentration both had shown similar behaviour. Maximum GABA yield of 6.55 mg/gds was observed with dextrose concentration of 80 g/l for co-culture whereas for *M. purpureus* it was 8.1 mg/gds with dextrose concentration of 120 g/l. This highlights the divergent effect of dextrose concentration on GABA yield for *M. purpureus* and co-culture.

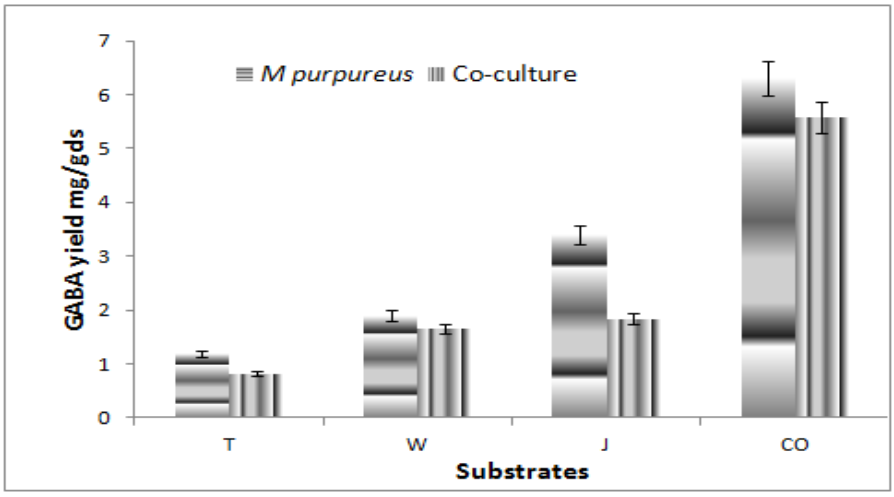

Fig 1 Screening of substrates for GABA production (T- Tamarind seed J- jack-fruit seed, W- Wheat bran, C- Coconut oil cake)

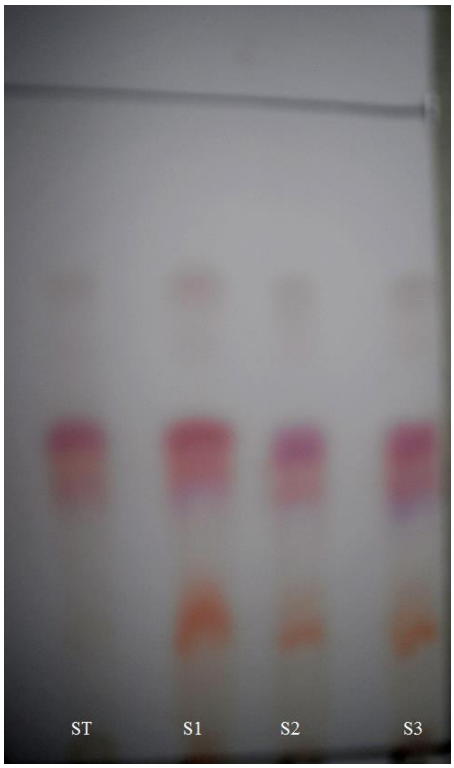

Fig 2 TLC chromatogram of the GABA identification (ST- Standard GABA, S1-*M. sanguineous*, S2-*M. purpureus*, S3-Co-culture of *M. sanguineous* and *M. purpureus*)

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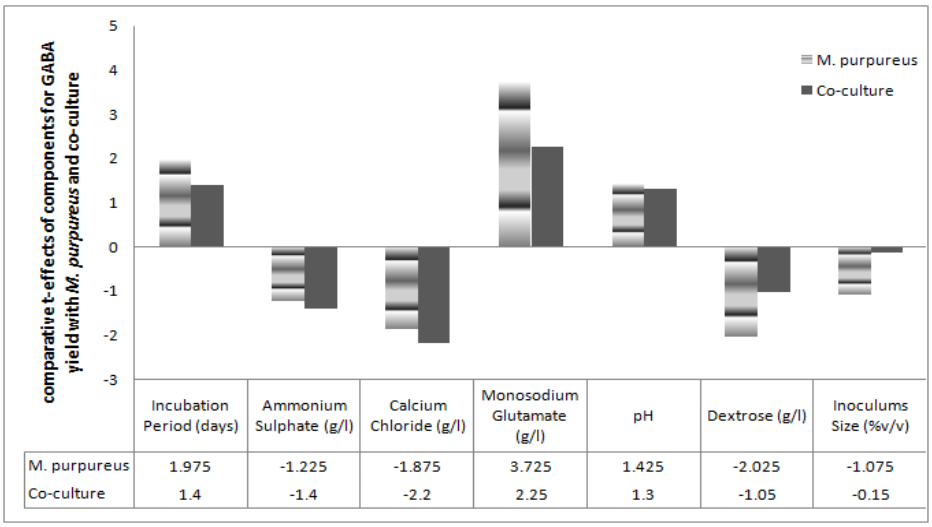

	Incubation Period (days)	Ammonium Sulphate (g/l)	Calcium Chloride (g/l)	Monosodium Glutamate (g/l)	pH	Dextrose (g/l)	Inoculums Size (%v/v)
M. purpureus	1.975	-1.225	-1.875	3.725	1.425	-2.025	-1.075
Co-culture	1.4	-1.4	-2.2	2.25	1.3	-1.05	-0.15

Fig 3 t- effects for the media components for GABA yield

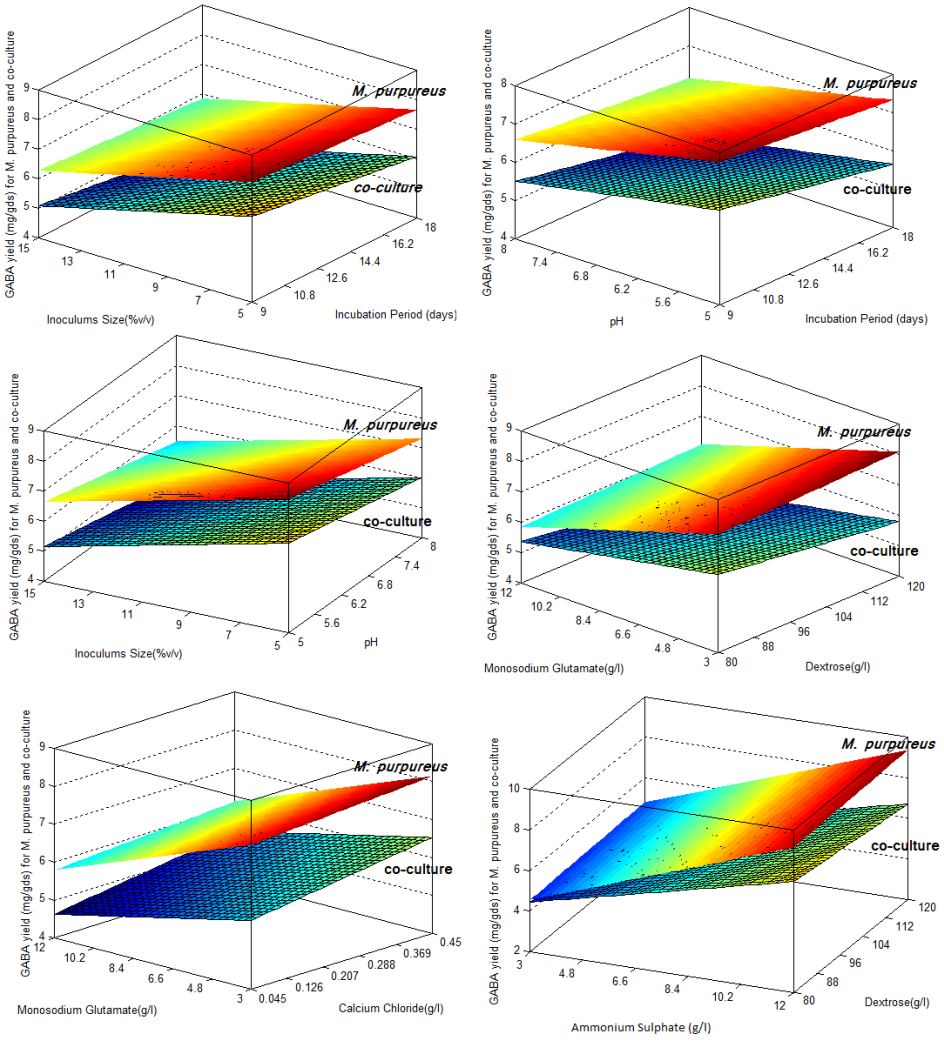

Fig 4 3D Response subplots for *M. purpureus* and Co-culture for all tested variables

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Table 1: Parameters and their levels with significance levels

Parameter Code	Parameter Name	Unit	Lower Level (-)	Higher Level (+)	*Monascus purpureus*			Co-culture of both strains		
					Σ (M_{i+})	Σ (M_{i-})	t-effect	Σ (M_{i+})	Σ (M_{i-})	t-effect
P_1	Incubation period	days	9	18	31.8	23.9	1.9	25.1	19.5	1.4
P_2	Ammonium sulphate	g/l	3	12	25.4	30.3	-1.2	19.5	25.1	-1.4
P_3	Calcium chloride	g/l	0.045	0.45	24.1	31.6	-1.8	17.9	26.7	-2.2
P_4	Monosodium glutamate	g/l	3	12	35.3	20.4	3.7	26.8	17.8	2.3
P_5	pH	-	5	8	30.7	25	1.4	24.9	19.7	1.3
P_6	Dextrose	g/l	80	120	23.8	31.9	-2.0	20.2	24.4	-1.1
P_7	Inoculums size	% v/v	5	15	25.7	30	-1.1	22	22.6	-0.2

The GABA yield for *M. purpureus* and co-culture increased with increase in the concentration of calcium chloride at low concentration of monosodium glutamate though the rate of increase was more with *M. purpureus* (Subplot 5 of Fig. 4). Maximum GABA yield of 8.14 mg/gds was obtained for *M. Purpureus* and 6.53 mg/gds for co-culture with 0.45 g/l concentration of calcium chloride and 3 g/l concentration of monosodium glutamate. For these variables also, a positive offset of 1.5 mg/gds in GABA yield was observed with *M. purpureus* as compared to the co-culture.

The GABA yield for *M. purpureus* remained almost constant with increase in the concentration of dextrose whereas it showed a slight decrease with co-culture (Subplot 6 of Fig. 4). Significant increase was observed in GABA yield with increase in concentration of ammonium sulphate for both *M. purpureus* as well as co-culture. The maximum value of GABA yield was found to be 9.36 mg/gds for *M. purpureu*s with 120 g/l concentration of dextrose and 12 g/l concentration of ammonium sulphate and 7.45 mg/gds for co-culture with 80 g/l concentration of dextrose and same concentration of ammonium sulphate. This demonstrates the positive effect of nitrogenous compound on the GABA yield.

For GABA biosynthesis, α-decarboxlation of L-glutamic acid (Glu) is a primary step and this reaction is catalyzed by glutamate decarboxylase (GAD) enzyme [19]. Obtained results suggest that appropriate amount of MSG can stimulate GABA production from *M. purpureus* and co-culture. Some authors have also reported that GAD activity could be regulated through glutamate in the substrate [9,20]. Further, ammonium sulphate, $CaCl_2$, dextrose and inoculum size had given negative t-value which suggests that glutamate decarboxyse (GAD) activity can be increased by sulphate and Ca ions but in certain amount of concentration. After which it might act as an inhibitor. GAD activity is rapidly stimulated by increasing the cytosolic Ca^{++} [21]. It is also observed there was a reduction in the yield of GABA at higher inoculum volumes. This was due to the fact that the higher inoculum volumes bring shorter lag phase resulting in lower GABA production. Lower inoculum volumes have a longer lag phase and hence result in a higher GABA production [22]. There was no significant increase observed in GABA yield with co-culture of these strains (*Monascus purpureus* and *Monascus sanguineus*). Some authors have reported higher yield in secondary metabolites with co-culture. Among these, Panda *et. al.* [13] had reported enhancement in lovastatin yield with co-culture of two selected *Monascus* strains (*Monascus purpureus* and *Monascus ruber*). Our results disagree with these authors for production of secondary metabolites with the co-culture of two used strains. In our previous study also, lovastatin yield was not appreciably higher with co-culture [23]. Hence it can be concluded that the co-culture technique may not always yield better results as the strains may not be compatible with each other as the case with *M. sanguineus* and *M. purpureus.*

CONCLUSION

Present study concludes that the production of γ-aminobutyric acid (GABA) from the co-culture of *M. purpureus* with *M. sanguineus* under solid state fermentation was found lesser as compared to their mono cultures. This endorses similar results obtained in our earlier work carried out on the production of lovastatin from this co-culture. This inhibitory characteristic of the strain with both the main compounds present was distinct in nature and differs with the idea of co-culture being a productive tool for the production of secondary metabolites.

REFERENCES

[1] Sun BS. [Dissertation for Master Degree.] Zhejiang Industry University, China, 2004; 40–55.
[2] Hagiwara H, Seki T, Ariga T. Journal of Bioscience Biotechnology and Biochemistry 2004; 68: 444-447.
[3] Miura D, Ito Y, Mizukuchi A, Kise M, Aoto H, Yagasaki K. Life Sciences 2006; 79:259–264.
[4] Chou LS, Weimer B. Journal of Dairy Sciences 1999; 82:23–31.
[5] Nomura M, Kimoto T, Someya Y, Furukava S, Suzuki I. Journal of Dairy Sciences 1998; 81 : 1486–1491.
[6] Aoki H, Uda I, Tagami K, Furuya Y, Endo Y, Fujimoto K. Journal of Bioscience Biotechnology and Biochemistry 2003a; 67: 1018–1023.
[7] Panatda J, Hataichanoke N, Saisamorn L, Toshisada S, Takeshi K, Griangsak C. World Journal of Microbiology and Biotechnology 2010; 26: 257–263.
[8] Qian L. Brazilian Journal of Microbiology 2013; 44(1): 183-187.
[9] Sun B S, Zhou L P, Jia X Q, Sung C K. African Journal of Biotechnology 2008; 7 (24):4544-4550.
[10] Huang J, Mei L, Wu H, Lin D. World Journal of Microbiology and Biotechnology 2007; 23: 865–871.

[11] Dhakal R, Bajpai V K, Kwang-Hyun B. Brazilian Journal of Microbiology 2012; 1230-1241.
[12] Dikshit R, Tallapragada P. International Research Journal of Biological Sciences 2013; 2(5): 59-67.
[13] Panda BP, Javed S, Ali Md. Food Bioprocess Technology 2010; 3: 373-378.
[14] Dikshit R, Tallapragada P. Frontiers in Life. Sciences 2015a; 8(2): 172-181.
[15] Ting Q, Haixing L, Yusheng C. African Journal of Biotechnology 2010; 9(50): 8679-8681.
[16] Zhang G, Bown W. Phytochemistry 1997; 44(6): 1007 1009.
[17] Kammoun R, Naili B, Bejar S. Bioresource Technology 2008; 99(13): 5602–5609.
[18] Ramachandran S, Singh S K, Larroche C, Soccol C R, Pandey A. Bioresource Technology 2007; 98: 2000–2009.
[19] Reed KL, Maclntyre JK, Tobet SA, Trudeau VL, Maceachern L, Rubin BS, Sower SA. Brain Behaviour Evolution 2002; 60(1): 1-12.
[20] Scott- Taggart CP, Van COR, Mclean MD, Shelp BJ. Physiology of Plant 1999; 106(4) : 363-369.
[21] Bouché N, Fromm H. Trends in Plant Sciences 2004; 9(3): 110-115.
[22] Donghua J, Hao J, Yan Y, Jiaheng H. European Food Research Technology 2011; 232 :541–547.
[23] Dikshit R, Tallapragada P. Journal of Food Science and Technology 2015b; 52(10):6679-6686.