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Efficiency of growth cultures of *Bacillus subtilis* with *Luffa cylindrica* used as carbon source

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The Tori (*Luffa cylindrica* L.) also known as Sponge Gourd (SG) is cultivated in tropical and sub-tropical regions of the world. Its fruit is useful as vegetable while rest has been remained non-edible agro-waste material. Present study was aimed to examine cell growth attributes of *Bacillus subtilis* (k1) in sub-merged fermentation cultures supplemented with Tori as carbon source. The fermentation cultures with 12.5 % extracts (v/v) of rotten Tori fruit were maintained i.e. LB_o [TY-medium: 10.0 g l^{-1} trypton, 5.0 g l^{-1} NaCl, 5.0 g l^{-1} yeast extract in dH_2O (w/v)], LB_1 ($^1/_8$ LB_0), LB_2 (LB_1 + peels of Tori) and LB_{2a} (LB_1 + peeled Tori) for 18 hrs of incubation. Cell growth and sugar contents were observed significantly higher in LB_2, while reducing sugars and phenolic contents in LB_{2a} culture supernatants. The activities of hydrolytic enzymes like as xylanases and pectinases also observed higher in LB_0 and LB_2 cultures ($p \geq 0.05$). Further significant increase in their activities observed in the cultures added with 5.0 m mol^{-3} cadmium (Cd^{2+}). In conclusion, Tori are rich with various phyto-metabolites to be the useful agro-nutrient rich fermentation substrate for the production industrial enzymes as well as other secondary metabolites.

Keywords: *Bacillus subtilis*, sponge gourd, antioxidants, xylanases, pectinases, metal stress.

INTRODUCTION

The Tori (local name) or sponge gourd (*Luffa cylindrica* L., 2n = 26) member of *Cucurbitaceous* is cultivated mostly in tropical and subtropical regions (Wu et al. 2016). Its immature fruit is used as vegetable, while fully-grown stuff and whole plant remained unmanaged wastage. Biochemically, sponge gourd is rich with many bio-activators possesses with anti-inflammation, anti-bacterial, anti-fungal, anti-myocardial properties including anthocyanins (0.5 mg g^{-1}), flavonoids (17.94 mg g^{-1}), glycosides, triterpenoid, saponins, cardiac glycosides, carbohydrates (29.51 %), alkaloids, proteins (33.55 %) fibers (6.47 %), fat (22.17 %), phenolics (20.74 mg g^{-1}), ascorbic acid (1.2 mg g^{-1}) and tannins (37-55%) comparable in concentrations from leaves to seed (Irshad et al. 2010; Osuagwu and Edeoga, 2014; Tripathy et al. 2014). The Tori plant body contains 10 % lignin, 30 % hemi-cellulose and 60 % cellulose (Rowell, 2000). Among the in-organic, *L. cylindrica* is rich with mineral contents including Mg, Na, Ca, K, Cu, Fe, Mn and Zn). This above nutritional potential of Tori is also very important source of vegetable protein, which has remained equally beneficial nutrition for both animal and human (Dairo et al. 2007).

The Tori are rich with growth inducing valuable agro-nutrients and being supportive to maintain in-vitro cultures (Tanobe et al. 2005). Recent studies reported that it has been used in bioreactor for production of ethanol from raw cassava starch (Roble et al. 2003). This sponge gourd has also been used in the nutrient cultures to prepare hepatocyte cell line of human (Chen et al. 2003). It has good performance in biofilm formation when used as solid state-substrate with maximum capabilities to adsorb nitrifying organic and

inorganic agents (de Sousa et al. 2008). Being a suitable natural matrix has been used for bio-sorption of cadmium (Cd) and lead (Pb) metals successfully (Saeed and Iqbal, 2013: Boudechiche et al. 2016). Such compact and rich with lignocellulosic plants could be the major component in various wastages from household to agriculture including vegetable markets. It may be the staple source of fermentation substrate (Behera et al. 2014).

The physio-chemical conditions including the structural factors of complex agro-waste based substrates hinder the biocatalyst based hydrolysis for their conversion to microbial-fuels. In past decade, a series of chemical based pre-treatment methods have been employed for fermentation biomass preparations which are increasing the cost of fermentation including the 10 % loss of yields from lignocelluloses (Gräsvik et al. 2014; Kucharska et al. 2018). Meanwhile, these loses are increasing the costs of bio-productions. The biodegradation of energy-rich agro-wastes by microorganisms has great economic deal over physio-chemical approaches (Abd-Elsalam and El-Hanafy, 2009; Sánchez, 2009; Bansal et al. 2015; Belal et al. 2018). In agro-wastes, about 60 % of plant biomass is lignocellulose. It is comprised on renewable cellulose and hemicelluloses, which are linked with lignin covalently (Tengerdy and Szakacs, 2003). The *Bacillus* spp., are diversified fermentation organism that have proven the best producer of many hydrolyzing extra-cellular enzymes including xylanases (Bansal et al. 2014; Mullai et al. 2010), cellulose (Mawadza et al. 2000), lipases (Al-limoun, 2019), pectinases (Kashyap et al. 2003) and strain of bioflocculant production (Goldern et al. 2019) etc.

By keeping in view of aforementioned facts, the present study was planned to utilize the agro-industrial wastes of Tori (*Luffa cylindrica* L.) as fermentation substrate. The *Bacillus subtilis* (k1) was cultured to analyze the potential of substrate on cell growth and other extra-cellular productions including enzymes. These extra-cellular bio-productions ultimately will effect on the decrease of their costs positively.

MATERIALS AND METHODS

Collection of plant materials

For present experiment, agro-waste of Tori or sponge gourd (*Luffa cylindrica* L.) fruit was used as fermentation substrate. It is a well-known fruit and used as vegetable. It was collected from Korai Vegetable and Fruit Market of the city. The collected stuff was further prepared to use in the lab.

Preparation of fermentation substrate

The collected stuff of rotten Tori fruit was washed with running tap-H_2O and dried with toilet-towels. The peels of the fruit were peeled-off with fine knife. Both peels and peeled material were used to prepare their filtrates. The 100 g of each were taken in the container of electric-grinder and then 100 ml of d H_2O was added. The mixture was grinded in grinder until homogenous mass of appeared. Both semi-solid plant masses were filtered with muslin-cloth at the room temperature. These fresh-filtrates were used as fermentation substrate.

Preparation of Tori based fermentation cultures

The Tori agro-waste based fermentation cultures were prepared in liquid broth nutrient LB_o (1.0 % Bacto-trypton, 0.5 % yeast extract, 0.5 % NaCl and pH 7.0) medium. Exactly 12.5 % Tori's extracts (filtrate of peels and peeled stuff) sustained low concentrated (LB_1: ⅛ strength LB_o) medium, while LB_0 medium was considered as positive and LB_1 as nutrient deficit control medium (Table 1). These fermentation cultures were sterilized under standard autoclave-conditions (121°C, 20 min, 15 psi) and it was used when cool down to room temperature.

Preparation of inoculum of fermentation organism

The glycerol stock of *Bacillus subtilis* (k1) was used to prepare for its master-culture. The wire-loop was dipped in its stock and inoculated in 2 ml LB_0 broth (Table 1). After over-night incubation at 37°C with 250 rpm shaking (de Vries et al. 2004), its 0.1 ml was sub-cultured in 5 ml LB_0 medium. After 30 min of incubation, it was used as a master culture to inoculate all other culture as shown in table 1.

Harvesting of *Bacillus* fermentation culture

After incubation of bacterial fermentation cultures for 18 hrs under *Bacillus* specific growth conditions (37°C with 250 rpm shaking), their OD600 was taken. After that cultures were harvested and their supernatants were separated with centrifugation for 10 min at 7,000 rpm. It was transferred to aseptic-dark-colored bottles and its pallets were freezed at -20°C. Culture-supernatant were stored at 4°C until next use for different biochemical and enzyme activities.

Biochemical analysis of *Bacillus* culture

The collected culture supernatant was subjected for analysis of various biochemical productions. Like as, total protein contents were measured by taking 1.0 ml culture supernatant in glass test-tube and 2.50 ml alkaline Copper reagent mixed thoroughly. After 10 minutes, 0.25 ml folin-ciocalteu reagent (1:1 dH_2O v/v) was added. By keeping 30 minutes at room temperature, its absorbance was read against blank at OD750 (Lowry et al. 1951). Total sugars were quantified by mixing 0.5 ml supernatant with 2.5 ml H_2SO_4 (conc.) than 0.05 ml phenol (80 %) mixed. After 15 minutes, OD485 was read (Montgomery, 1961). The reducing sugars were also measured in culture supernatant by mixing its 1.0 ml with 1.0 ml 2,6-dinitrosalicylic acid (DNS) in test tube. Mixture was heated for 5 minutes at H_2O-boiling point in H_2O-bath then its OD540 was taken (Miller, 1959). Similarly, phenolics were also measured (Yasoubi et al. 2007) in cultures by taking its 0.2 ml in test tube mixed with 1.0 ml folin-ciocalteu than 0.8 ml Na_2CO_3 was added. After keeping the mixture at room temperature for 30-minutes, OD765 was taken. The flavonoids and antioxidants were also measured in the culture supernatant by following the methods of (Li et al. 2007) and (Djeridane et al. 2006) respectively.

Extra-cellular enzyme activities under cadmium (Cd^{2+}) stress

Culture-supernatant of *Bacillus subtilis* (k1) subjected as crude enzyme for analysis of xylanases and pectinases activities. The *xylanases* activities determined by taking 1 ml supernatant mixed with 1 ml substrate (0.5 % xylose). After incubation for 15 min at 60°C, 2 ml DNS added then OD540 was noted (Bailey et al. 1992). Similarly, pectinases (Miller, 1959) were assayed by mixing 0.05 ml supernatant as crude enzyme with 1.0 ml substrate [0.25 % pectin in 50 mM citric-citrate buffer (pH 3.5)] than 1.5 ml DNS added (Miller, 1959; Merín et al. 2011). The thermos-ability of both xylanases and pectinases activities also determined under 5.0 m mol^{-3} per liter concentration of cadmium (Cd^{2+}) metal ions stresses. These above same reactions were also conducted and incubated at 37°C for 30 min.

Statistical analysis of data

The data of present study was computed for culture significance (ANOVA - analysis of variance and DMR - Duncan's Multiple Range test at 5 % probability level with "COSTAT" package by *CoHort* Software, Berkeley, USA (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Cell growth and effects of carbon sources

The growth of cells and organisms require energy and carbon compounds are rich-sources of essential energy for their movement and reproduction (Hodges, 2010; Jones, 2012). Agro-wastes are readily available as ligno-cellulosic rich and economic carbon sources. In present study, effects of Tori or sponge gourd (*Luffa cylindrica* L.) fruit used as carbon sources on *Bacillus subtilis* (k1) growth was assessed in the culture harvest taken after 18 hrs of incubation and shown as in table 1. The optimal standard *Bacillus* spp., growth culture is TY-medium (LB_o; Table 1) with OD600 reached to 1.555±0.029. It was comparable to its cultures maintained on peels of Tori fruit (LB_2) and peeled Tori fruit (LB_{2a}) used as carbon source with OD600 observed to 1.568±0.055 and 1.528±0.081 respectively (Table 2). The glucose is preferred nutrient source for fermentation organisms including *B. subtilis* (Meyer et al. 2014), while the glucose production ability of the organisms depends on type of secreted hydrolyzing enzymes and available substrate in the fermentation medium (Nigam and Singh, 1995; La Grange et al. 2010; Kampen, 2014).

Table 1: Preparation of agro-waste based fermentation medium for *Bacillus subtilis* (k1) supplemented with agro-wastes of Tori (*Luffa cylindrica* L.) as substrate.

#s.	Medium	Composition of medium
01	LB_o	**TY-medium:** Bacto-trypton (1.0 %), NaCl (0.5 %), yeast extract (0.5 %) in distilled-H_2O (w/v)
02	LB_1	⅛ LB_o in dH_2O (v/v)
03	LB_2	LB_1 + 12.5 ml peels of tori + 12.5 ml distilled-H_2O (12.5 %, v/v)
04	LB_{2a}	LB_1 + 12.5 ml peeled tori + 12.5 distilled-H_2O (12.5 %, v/v)

Note: The final concentration of each extract adjusted to 12.5 % in 50 ml final volume of medium. Each c*ulture maintained with 4 replicates with 50 ml final volume.*

Table 2: Cell growth of *Bacillus subtilis* (k1) and its related extra-cellular attributes in cultures supplemented with agro-wastes of Tori (*Luffa cylindrica* L.) as fermentation substrate.

#s.	Characteristics	LB	Tori (*Luffa cylindrica* L.)			*F*-significance
		LB_0	LB_1 (⅛LB_0)	LB_2	LB_{2a}	
01.	Cell growth (OD_{600})	1.555±0.029^b	0.260±0.009^c	1.568±0.055^a	1.528±0.081^{bc}	159.5***
02.	Total sugars (mg ml^{-1})	22.18±0.134^b	8.636±0.290^c	30.06±0.087^a	29.53±0.258^a	2261***
03.	Reducing sugars (mg ml^{-1})	6.058±0.116^c	2.927±0.241^d	8.801±0.116^{ab}	9.0230.1385^a	80.32***
04.	Total proteins (mg ml^{-1})	7.299±0.059^c	2.551±0.220^d	9.319±0.234^a	8.669±0.129^b	302.3***
05.	Ascorbic acid (mg ml^{-1})	3.735±0.112^c	1.990±0.064^d	4.855±0.071^a	4.800±0.073^a	267.1***
06.	Proline (mg ml^{-1})	0.057±0.002^a	0.026±0.001^b	0.055±0.001^a	0.057±0.002^a	120.3***
07.	Phenolics (mg ml^{-1})	2.833±0.160^b	2.833±0.132^b	5.778±0.143^a	5.833±0.289^a	80.08***
08.	Flavonoids (mg ml^{-1})	0.341±0.018^b	0.176±0.010^c	0.474±0.005^a	0.498±0.011^a	150.4***
09.	Antioxidants (mg ml^{-1})	0.912±0.028^b	0.343±0.021^c	1.847±0.022^a	1.822±0.020^a	1034.6***

The collected data is mean of 4-replicates of all treatments or cultures [mean ± SE (n = 4)]. The Duncan's Multiple Range test was performed at p≥0.05 level of ANOVA significance.

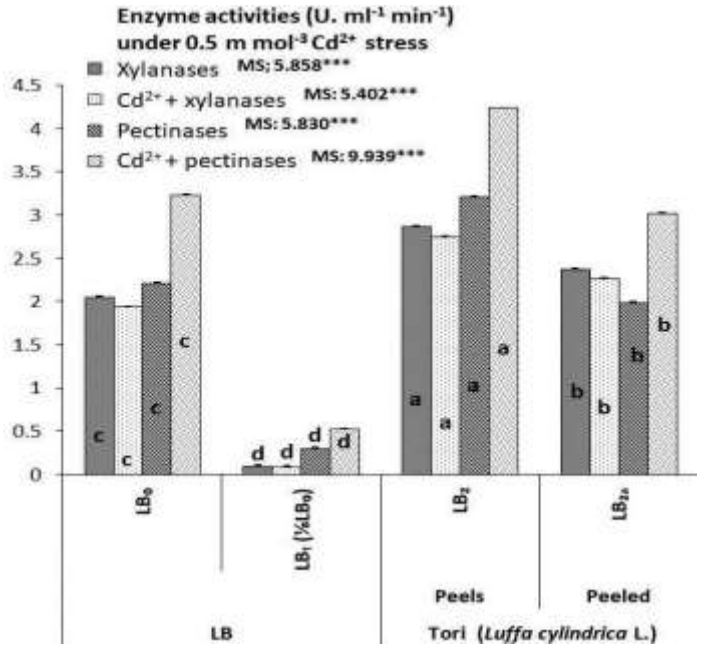

Figure 1: Effect of cadmium (Cd^{2+}) metals on crude-enzyme activities of *Bacillus subtilis* (k1) cultures supplemented with agro-wastes of Tori (*Luffa cylindrica* L.) as fermentation substrate.

Productions of various extra-cellular metabolites

With rapid increase in cell multiplication rates on different cultures supported with control to agro-waste based carbon sources, various extra-cellular metabolites were increased. Like as total sugars and reducing sugars were higher in LB_{2a} culture supernatants which were harvested after 18 hrs of incubation than the control cultures (Table 1 & 2). Similarly other metabolites, total protein contents and antioxidants of fermented substrates were higher in LB_2 and LB_{2a} cultures. The *Bacillus subtilis* is serving as single cell protein secretor. In other studies it has been reported that protein contents increases when ligno-cellulosic rich agro-wastes are used as fermentation substrate (Ofuya and Nwajiuba, 1990; Shabeb et al. 2010; Zerdani et al. 2004). The other antioxidants also observed with differential concentrations among the culture supernatants.

Maximum proline and ascorbates were observed among the agro-wasted based cultures LB_{2a} and LB_2 supernatants respectively ($p \geq 0.05$), while phenolics and flavonoids were higher in LB_{2a} cultures (Table 2). With increase in culture time, the production of metabolites in the cultures increases, which lead to develop specific detrimental effects on culture growth as well as extra-cellular bio-productions (Misawa, 1994; Sravanthi et al. 2016). For mitigation of the developed stress, the cell triggers the endogenous free proline and ascorbate accumulation in different organisms (Wood et al. 2001; Shivanand and Mugeraya, 2011).

Extra-cellular enzymes productions

The results of xylanases and pectinases activities in the control as well as Tori agro-waste based submerged fermentation (SSF) culture supernatants of *B. subtilis* (k1), which were harvested after 18 hrs of incubation (Fig 1). The maximum pectinases and xylanases activities among the culture supernatants were observed in LB_2 (Tori-peels) cultures than other LB_o (TY-medium) and LB_{2a} (peeled-Tori). The availability of xylan and pectin in the culture triggers the production of their respective hydrolyzing enzymes (xylanases and pectinases) like as the richness of cellulases activities in cellulose rich extracts. Even hemicellulose are barrier for cellulases access to cellulose that's why its hydrolysis is essential for efficient agro-waste saccharification (Zimbardi et al. 2013). The above fact is due to that cultured organism is producing all enzymes required to digest the biomass used for its growth (Kumar et al. 2008; Znameroski et al. 2012).

Cadmium (Cd^{2+}) stresses on enzyme activities

According to the figure 1, it shows the activities of xylanases and pectinases in agro-biomass based culture supernatants of *B. subtilis* (k1), which were harvested after 18 hrs of incubation. With the application of 0.5 m mol^{-3} $CdCl_2$ (Cd^{2+}) stress, its significant effect on enzyme activities was observed (Fig 1). The xylanase activities were decreased, while pectinases showed increase in its activities in all culture supernatants. Such inhibitions as well as enhancements are adopted naturally by the indigenous microorganism for biotransformation of toxic metals (Benammar et al. 2015; Voica et al. 2016). It is specific required industrial features for attraction of commerce community. Among the saline bacteria, it is widespread character (Nieto Gutiérrez et al. 1989; Cánovas et al. 2000; Amoozegar et al. 2005). For xylanases, Cd^{2+} has shown inhibitory effect, while remains enhancer for pectinases activities. It means that cadmium metal ions (Cd^{2+}) are playing vital role to keep active confirmation to stimulate pectinases activities (Beg and Gupta, 2003).

The main applications of hydrolytic enzymes are to utilize complex agro-waste biomass conversion into simple absorbable carbon molecules by the microbes. Various groups have studied in order to test saccharification potential of celluloses for rice straw (Ball and McCarthy, 1988) or other cellulose rich organic biomasses (Prasad et al. 2012). In the last decade, various typed agro-biomass have been utilized for sustainable productions of secondary metabolites enzymes and biofuels and chemicals (Puri and Abraham, 2016; Voloshin et al. 2016; Kumar et al. 2017; Vellaichamy and Saxena, 2020). At the moment, present study is carried out to evaluate the potential of cellulosic extract of Tori plant fruit with *Bacillus subtilis* (k1) for bio-production of metabolites and saccharification enzymes like as xylanases and pectinases.

CONCLUSION

The in-vitro cellular productions are considered economic, natural and safe than via chemical methods with excess energy. The utilization of low cost lingo-cellulosic rich agro-wastes could fulfill the economic energy needs. Tori-wastes are full with carbon and nitrogen sources for *Bacillus subtilis,* which has significant role in industrial production including various enzymes. Like as the cultures supplemented with Tori's wastes (LB_2 and LB_{2a} cultures) shown comparative ($p \geq 0.05$) cell growth, production of metabolites and important industrial enzymes. Present work could be helpful in future for selection of energy-rich part of plant wastes for the production of maximum extracellular metabolites and its subsequent fermentation organism.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Author 1 and 2 has carried out the present research work, while Author 2 designed the objectives and guided throughout the study. Author 3 tabulated the data and statistical analysis. Author 4 & 5 have pre-submitted review this present manuscript critically.

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