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INFLUENCE OF CARBON, NITROGEN AND METAL SALTS ON α-AMYLASE PRODUCTION BY *BACILLUS MEGATERIUM* KLMI 4 USING AGRO-WASTES

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ABSTRACT

Maximum activity of α-amylase was observed with fructose 375.0 IU, closely followed by that with supplementation of maltose (360 IU/ml). Several sugars like dextrin 275.0 IU, glucose 170.0 IU, glycerol 178.0 IU, trehalose 140 IU and xylose 148 IU and medium amended with starch 210 IU caused lower enzyme activity but higher than that in the controls: The non-metabolizable sugars like arabinose, raffinose, meso-inositol, sucrose and galactose do not support α-amylase production as indicated by enzyme activities much lower than that in the control, whereas with lactose lower enzyme activity is observed as the organism is basically a non-lactose fermenter. Addition of peptone proved to be beneficial for enzyme production, enzyme yield at 48 h increased gradually as peptone concentration increased upto 1.5% and thereafter it decreased as peptone concentration increased, the enzyme yield being suppressed at 3.5 and 4.0% peptone concentrations. The highest yield of α-amylase 214.0 IU was obtained with 1.5% peptone**.** Amongst all the organic nitrogen sources supplemented, peptone was observed to be most beneficial for α-amylase production with an yield of 215.0 IU. Urea tended to suppress the yield of the enzyme. The order of beneficiality of the sources is in the following manner**:** peptone>beef extract>tryptone>yeast extract> hydrolysed casein>corn steep liquor and soluble casein. Ammonium bisulphate was observed to be most beneficial, α-amylase activity of 195.0 IU after 48 h of incubation period was recorded. Amongst other salts, only ammonium nitrate and ammonium sulfate were beneficial. Other salts were slightly suppressive. Amongst the metal salts supplemented, zinc sulphate, mangenese sulphate and ferrous sulphate were observed to exhibit suppressive action on α-amylase production by the bacterium. Other salts enhanced α-amylase production, maximum yield 198.0 IU being observed with calcium sulphate. The beneficial influence of these salts was observed to be in the following order : calcium sulphate>calcium chloride >magnesium sulphate >copper sulphate. The enzyme activity was 65.0 IU at 24 h and it rose sharply to 513.0 IU at 48 h, almost eight times as that at 24 h. Thereafter it declined very sharply, recording 160.0 IU and 35.0 IU at 72 and 96 h, respectively.

KEYWORDS: Bacillus megaterium KLMI 4, Alpha-amylase production, Agro-wastes (substrate), influence of carbon, nitrogen, metal salts.

INTRODUCTION

Sugars, the reduced forms of organic carbon and also the amino acids, are essential for the cells as energy sources and building blocks for the cells themselves. With this aspect as well as the desired product (in this instance α-amylase) different reduced sugars are supplemented separately or in combination into the fermenting medium to obtain its optimum and better yield.

Carbon and nitrogen sources play an important role in the process of α-amylase production by a microorganism. Hence, it was felt worthwhile to evaluate the influence of different sugars as C sources and various organic and inorganic N sources on α-amylase production by the organism under submerged fermentation of green gram husk.

Nitrogen sources also play an important role in the maintenance and activities of the bacteria and living organisms in general.

Various inorganic salts are known to influence growth and activity of microorganisms. Hence, studies were carried out to evaluate the influence of some nitrogen based and other inorganic salts (sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate and ammonium bisulphate) on the α-amylase production by the bacterium.

Some metal ions act as co-factors for microbial enzymes. It is also known that some microorganisms depend on calcium for their activities. Hence, studies were carried out to evaluate the influence of some metal ions and calcium salt on the potency of the microbe under consideration to produce α-amylase through SmF.

MATERIALS AND METHODS

Carbon Sources

The influence of different sugars was studied maintaining all the optimized physico-chemical parameters of the fermenting medium constant, i.e. *p*H at 8.0, temperature at 37^0 C, and inoculum size of $3x10^6$ cfu/ml. Hence, different sugars like fructose, meso-inositol, glucose, galactose, sucrose, arabinose, raffinose, lactose, maltose, dextrin, xylose, glycerol and trehalose as well as starch were individually and separately added to the green gram husk extract at concentration of 1 g/100ml with 0.5% NaCl. After submerged fermentation under optimized conditions, samples were withdrawn at intervals of 24 h and α-amylase activity was estimated as detailed earlier. The study was conducted in triplicate for each C source. Green gram husk without any sugars or starch served as the control.

Nitrogen Sources

Peptone, various other complex organic substrates like tryptone, yeast extract, beef extract, corn steep liquor, casein (both hydrolyzed and soluble) and urea have were used to observe their influence on bacterial activity.

1. Peptone Supplementation and α-Amylase Production

Initially, the peptone requirement of the organism was determined by adding different concentrations of peptone (0.5 to 4.0%, with intervals of 0.5%) to the extract and autoclave sterilized before inoculating the bacterial suspension. Maintaining the already optimized fermentation parameters as detailed earlier, SmF was conducted over a period of 72 h. Samples were collected every 24 h to estimate the α-amylase activity. Appropriate controls were maintained without Peptone supplementation.

2. Supplementation of other Organic Nutrients

Since 1.5% peptone was required for optimum α-amylase production, the influence of other organic nutrients (tryptone, yeast extract, beef extract, corn steep liquor, both hydrolyzed and soluble casein as well as urea) on α-amylase production by the bacterium was evaluated. These were added at concentrations of 1.5% individually and separately to the fermentation medium. Samples from the individual flasks were withdrawn every 24 h and α-amylase production was estimated. Appropriate controls were maintained without organic nutrients supplementation.

3. Supplementation of Inorganic Nutrients

Each of these salts (1% w/v) was dispensed into the fermentation medium separately and autoclave sterilized. The inoculated flasks were incubated in the same conditions as detailed earlier. Required samples were withdrawn aseptically at intervals of 24 h and α-amylase production was estimated. Appropriate controls were maintained without Inorganic Nutrients supplementation.

Supplementation of Metal Salts

The metal ions selected for the study were magnesium sulphate ($MgSO_4.7H_2O$), manganese sulphate ($MnSO_4.7H_2O$), copper sulphate ($CuSO_4$), zinc sulphate ($ZnSO_4.7H_2O$), ferrous sulphate ($FeSO_4.7H_2O$) and calcium chloride ($CaCl_2$) and calcium sulphate ($CaSO_4$). Each of these (1 g) was dispensed into 100 ml of the fermenting medium separately and autoclave sterilized before inoculating the fermenting organism. Then the flasks were incubated at 37^0 C and samples were withdrawn every 24 h from the flasks for enzyme estimation. Appropriate controls were maintained without metal salt supplementation.

Overall Influence of Nutrient Supplementation on α-Amylase Production

After establishing the optimum physico-chemical parameters for SmF of the fermenting medium as well as the different organic and inorganic nutrients, the combination of all optimized parameters was analyzed. For this purpose, the most influencing sugar (fructose, 1% w/v) as well as peptone (1.5%), ammonium bisulphate and calcium sulphate (each 1% w/v) were added to the green gram husk extract and autoclave sterilized at 121^0C for 15 min. After the fermenting medium cooled to room temperature, bacterial suspension (approximately $3x10^6$ CFU/ml) was inoculated into each flask aseptically and incubated in a rotary shaker incubator with 200 rpm at 37^0 C. The study was run in triplicate. Samples were withdrawn from each flask every 24 h and analyzed for α-amylase activity.

RESULT

Influence of Sugars on Enzyme Production

The results of the enzyme activity after 48 h of incubation are presented in Fig. 1.

Maximum activity of α-amylase was observed with fructose (375.0 IU/ml), closely followed by that with supplementation of maltose (360.0 IU/ml). Several sugars like dextrin (275.0 IU/ml), glucose (170.0 IU/ml), glycerol (178.0 IU/ml), trehalose (140.0 IU/ml) and xylose (148.0 IU/ml) and medium amended with starch (210.0 IU/ml) caused lower enzyme activity but higher than that in the controls: The non-metabolizable sugars like arabinose, raffinose, meso-inositol, sucrose and galactose do not support α-amylase production as indicated by enzyme activites much lower than that in the control, whereas lower enzyme activity is observed with lactose as the organism is basically a non-lactose fermenter.

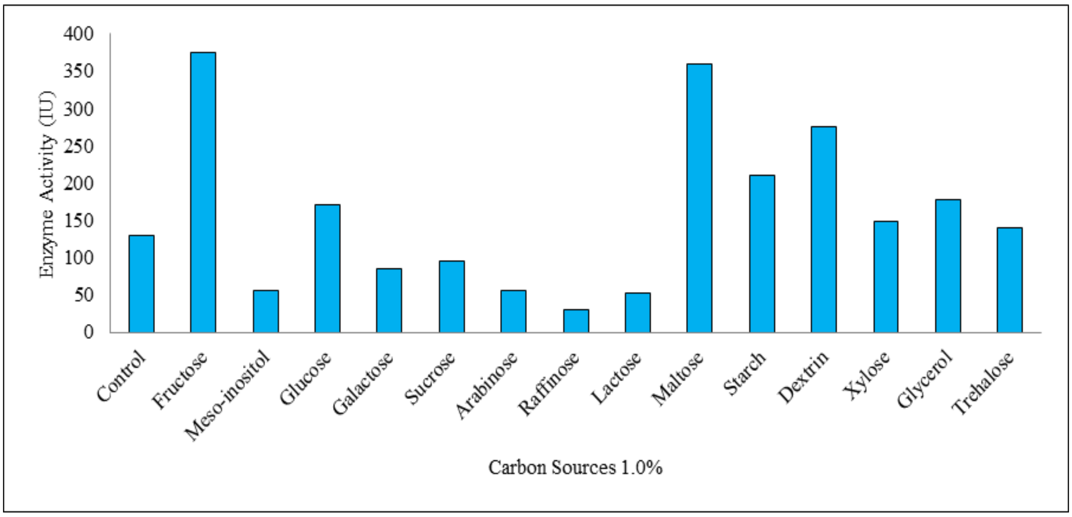

Fig. 1: Influence of Sugars on Enzyme Production.

Influence of Nitrogen Sources

1. Influence of Peptone on Enzyme Production

Addition of peptone proved to be beneficial for enzyme production, enzyme yield at 48 h increased gradually as peptone concentration increased upto 1.5% and thereafter it decreased with increase in peptone concentration; the enzyme yield was suppressed at 3.5 and 4.0% peptone concentrations. The highest yield of α-amylase (214.0 IU/ml) was obtained with 1.5% peptone. The results of the enzyme activity after 48 h of incubation are presented in Fig. 2.

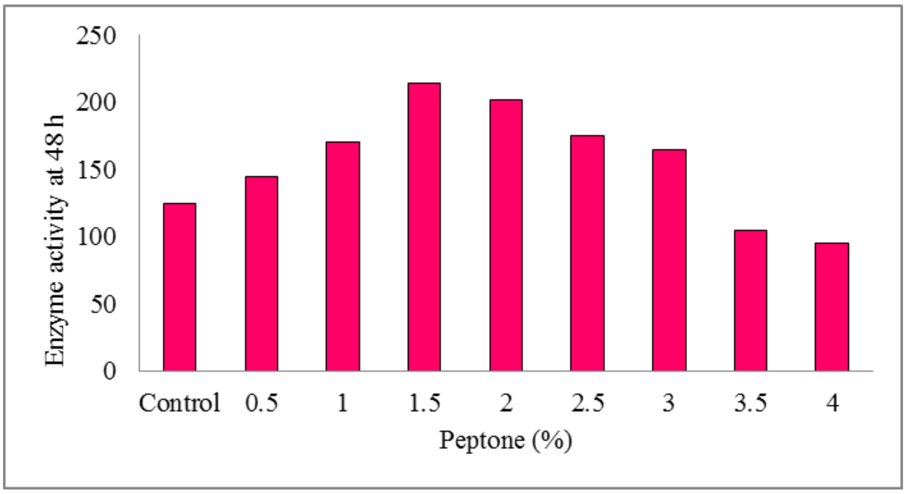

Fig. 2: Influence of Peptone on Enzyme Production.

2. Influence of Organic N Sources on Enzyme Production

The results of the enzyme activity at 48 h with different nitrogen sources are presented in Fig. 3.

Amongst all the organic nitrogen sources supplemented, peptone was observed to be most beneficial for α-amylase production with an yield of 215.0 IU/ml. Urea tended to suppress the yield of the enzyme. The order of beneficial influence of the sources is as follows: peptone>beef extract>tryptone>yeast extract> hydrolysed casein>corn steep liquor and soluble casein.

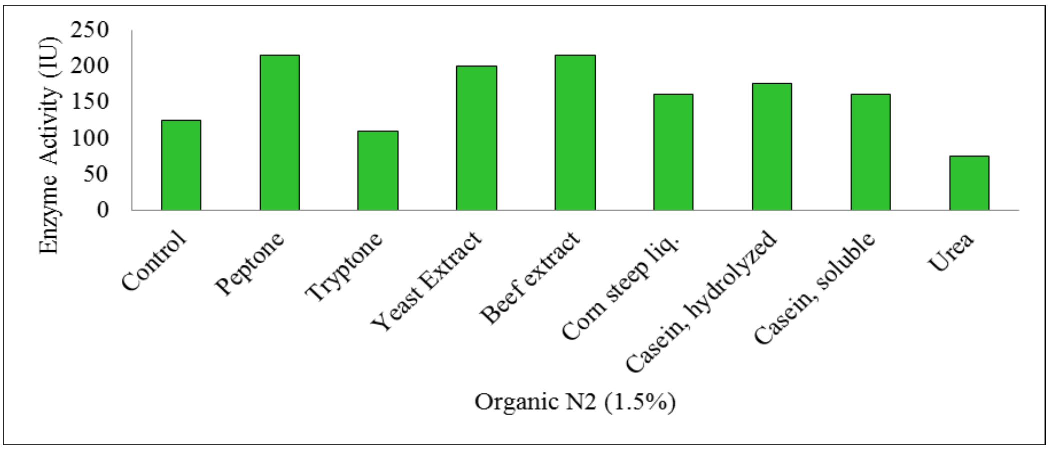

Fig. 3: Influence of Organic N Sources on Enzyme Production.

3. Influence of Inorganic Nitrogen Sources on Enzyme Production

The enzyme activity as influenced by different inorganic nitrogen sources was estimated after 48 h of incubation and the results are presented in Fig. 4.

Ammonium bisulphate was observed to be most beneficial, α-amylase activity of 195.0 IU/ml after 48 h of incubation period was recorded. Amongst other salts, only ammonium nitrate and ammonium sulphate were beneficial. Other salts were slightly suppressive.

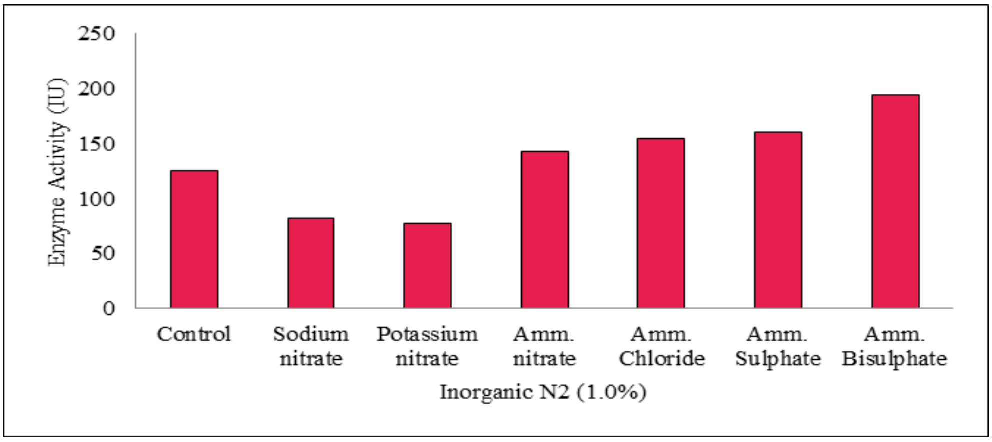

Fig. 4: Influence of Inorganic Nitrogen Sources on Enzyme Production.

Influence of some Metal Salts on Enzyme Production

The enzyme production as influenced by different metal salts was estimated after 48 h of incubation and the results are presented in Fig. 5.

Amongst the metal salts supplemented, zinc sulphate, mangenese sulphate and ferrous sulphate were observed to exhibit suppressive action on α-amylase production by the bacterium. Other salts enhanced α-amylase production, maximum yield (198.0 IU/ml) being observed with calcium sulphate. The beneficial influence of these salts was observed to be in the following order : calcium sulphate>calcium chloride >magnesium sulphate >copper sulphate.

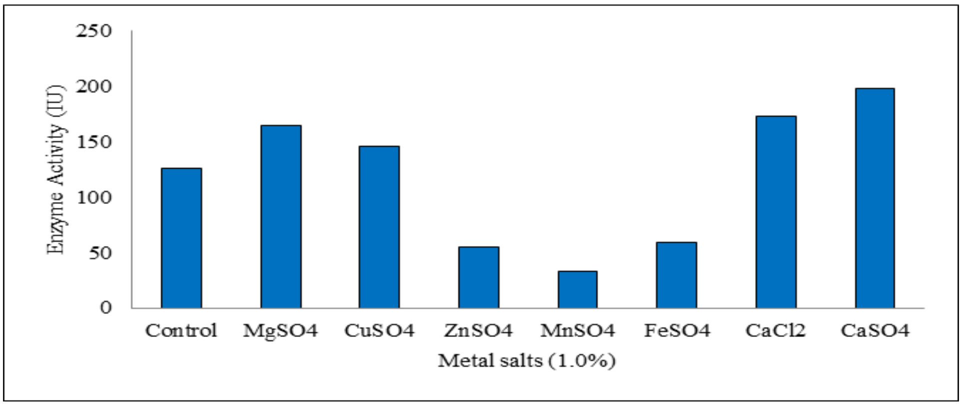

Fig. 5: Influence of Some Metal Salts on Enzyme Production.

Influence of Combination of all Optimized Conditions on α-Amylase Production

After all the physico-chemical parameters regulating the submerged fermentation process as well as the supplementary sugars, organic and inorganic nitrogenous sources and metal salts were optimized, it was considered worthwhile to evaluate the combiation of their optimum levels on the yield of α-amylase by the bacterium. Hence, fermentation studies were conducted with the following conditions : *p*H, 8.0; temperature, 37^0 C; and inoculum size, $3x10^6$ cfu/ml, fructose, 1.0%; peptone, 1.5%; ammonium bisulphate, 1.0% and calcium sulphate, 1.0%. The incubation period was 96 h. The results are presented in Fig.6.

The enzyme activity was 65.0 IU/ml at 24 h and it rose sharply to 513.0 IU/ml at 48 h, almost eight times as that at 24 h. Thereafter it declined very sharply, recording 160.0 IU and 35.0 IU/ml at 72 and 96 h, respectively.

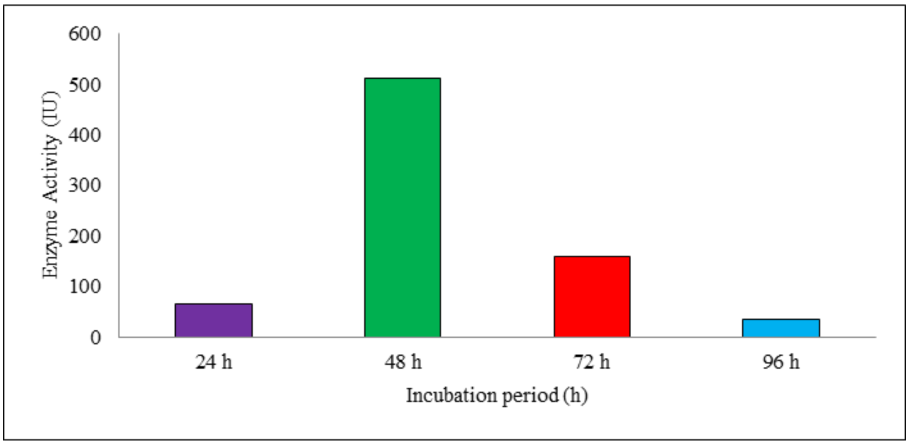

Fig. 6: Influence of Combination of all Optimized Conditions on α-Amylase Production.

Table 1: Use of different agro-wastes for microbial α-amylase production.

B. subtilis	Brewery wastes	Blanco *et al.* (2016).
B. polymyxa NCIM 2539	Orange peel waste (SmF)	Tripathi *et al.* (2017).
B. subtilis	Wheat bran (SmF)	Irfan *et al.* (2016).
B. subtilis MTCC 7524, *B. licheniformis* MTCC 7445	Dairy sludge (SSF)	Reya and Roy (2015).
B. subtilis ATCC 6633	Wheat bran (SSF)	Maity *et al.* (2015).
Bacillus sp. R2	Pasta cooking water (Smf)	Choubane *et al.* (2015)
B. licheniformis	Flours of wheat, barley, corn & gram, husks of moong, soyabean & arhar, oil cakes of mustard & coconut, rice bran, wheat bran, peels of potato, sweet potato & banana, sugarcane bagasse (SSF)	Singh *et al.* (2014).
Bacillus sp. CFR 67	Wheat bran, rice bran (SmF)	Sreekanth *et al.* (2013).
B. cereus MTCC 10202	Brans of wheat, maize, corn, millet, rice, green gram & black gram, cassava peel powder, oil cakes of cotton seed, coconut, sesame & ground nut. (SSF)	Kalaiarasi and Parvatham (2013)
Paenibacillus amylolyticus	Brans of wheat, rice & maize, rice husk. (SSF)	Ikram-Ul-Haq *et al.* (2012).
A. flavus	Corncob, rice bran, rice straw, sugarcane bagasse (SSF)	Padmini *et al.* (2012)
A. niger	Banana peel (SSF & SmF)	Krishna *et al.* (2012).
A. niger	Brans of rice, wheat, & black gram, oil cakes of gingelly & groundnut (SSF)	Suganthi *et al.* (2011).
Penicillium expansdum MT-1	Loquat kernels (SSF)	Erdal and Taskin (2009)
A. flavus	*Cocos nucifera* meal (SmF)	Arunsasi *et al.* (2010).
Aspergillus oryzae	Coconut oil cake (SSF)	Ramachandran *et al.* (2004).
B. subtilis (CBTK 106)	Banana waste (SSF)	Krishna and Chandrasekaran (1996).
A. awamori MTCC 9997	Cassava peel powder (SSF)	Kalaiarasi and Parvatham (2013).
Gliomastix indicus	Wheat bran, rice bran, mustard oil cake (SSF)	Mishra *et al.* (2016)
B. circulans ATCC 4516	Rice bran	Serin *et al.* (2012)
B. subtilis	Palm empty fruit bunch fibres, rice straw (SSF)	Huzairy and Khairiah (2012).
Lichthelma ramosa	Savannah fruit wastes	Silva *et al.* (2013).
B. mura	Cassava waste (SSF)	Vijayan *et al.* (2015).
B. subtilis	Banana waste (SSF)	Unakal *et al.* (2012).

Bacillus sp. PS-07	Wheat bran +soybean meal suppl. to glycerol (SSF), brans of corn and rice.	Sodhi *et al.* (2005).
A. oryzae NRRL 6270	Spent brewing grain (SSF)	Francis *et al.* (2003).
B. coagulans	Maize bran, gram bran, mustard oil cake (SSF)	Babu and Satyanarayana (1995).
B. subtilis	Rice husk (SSF)	Baysal *et al.* (2003).
A. flavus	*Amaranthus* grains (SSF)	Vishwanathan and Surlikar (2001).
B. subtilis PF1	Feather meal, potato peel, rape seed cake (SmF)	Bhange *et al.* (2016).
Thermomyces lanuginosus	Wheat bran (SSF)	Kunamneni *et al.* (2005).
A. flavus AUMC 11685	Mandarin (Icitrus *reticulata)* peel (Smf)	Ali *et al.* (2017).
B. subtilis	Banana peels	Paul and Sumathy (2013)
Achromobacter xylosoxidans	Straws of rice, cone, ragi & millet, sugarcane bagasse (SmF)	Mahalakshmi and Jayalakshmi (2016).
B. cereus MTCC 1305	Wheat bran, rice flakes (SSF)	Anto *et al.* (2006).
Bacillus sp.	Rice husk, rice bran, rice polish, defatted soybean meal, sunflower meal, cottonseed meal (SSF)	Irfan *et al.* (2011).
B. subtilis RSKK96	Wheat bran, rice husk, lentil husk, cotton stalk, coarse meals of corn & millet (SS)	Akcan *et al.* (2012).
B. licheniformis	Wheat bran, Corn steep liquor (SSF)	Niaz *et al.* (2010).
Bacillus sp. SMIA-2	Whey protein concentrate, corn steep liquor (SmF)	Correa *et al.* (2011).
B. subtilis MAFE 11809	Rice bran (SSF)	Rameshkumar and Sivasudha (2011).
Anoxybacillus flavithermus SO-13	Husks of rice, banana, melon, water melon, & pistachio, wheat bran, maize oil cake (SSF)	Ozdemir *et al.* (2015)
B. cereus	Cow dung	Vijayaraghavan *et al.* (2015)
Bacillus sp.	Peels of orange & pomegranate (SmF)	Prameela *et al.* (2016)

DISCUSSION

In the economization of the process by supplementation of different carbon and nitrogen sources as well as metal salts to the green gram husk extract, it has been observed that much higher α-amylase yield could be obtained. When all the optimized conditions of process variables, sugar, organic and inorganic nitrogen sources as well as metal salts were maintained, the α-amylase yield with green gram husk extract was much higher (513 IU/ml) compared to 395 IU/ml obtained with basal medium. As such an almost 30% higher yield of the enzyme is obtained.

The agro-wastes have been used to economize the process of α-amylase production successfully through SSF and SmF processes and an attempt has been made to list them in the Table. 1.

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