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Original Article

Optimization of medium and substrate for CMCase production by *Bacillus subtilis*-BS06 in submerged fermentation and its applications in saccharification

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MA, ZH: performed experiments, **MI, QS:** conceived study designed, **AR, MN, HAS**: drafted manuscript.

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Submerged fermentation
Saccharification

Abstract
In the present study a strain of *Bacillus subtilis*-BS06 was cultivated in submerged fermentation using agricultural wastes as substrates for carboxymethyl cellulase (CMCase) production under static and with agitation speed of 140 rpm. The whole fermentation experiments were carried out in 250ml Erlenmeyer flask. Different agricultural wastes such as sugarcane bagasse, wheat straw, rice husk, defatted soybean meal, corn cobs and wheat bran were exploited for CMCase production using different media (medium 1 (M1), medium II (M-II), medium III (M-III) & medium IV (M-IV)).Of all these tested substrates, sugarcane bagasse was found to be the most suitable substrate for CMCase production. The effect of shaking on the production of CMCase demonstrated that sugarcane bagasse gave maximum CMCase activity (8.0 ± 0.32 IU) after 48h of fermentation period at 37^{o}C with agitation speed of 140 rpm by using medium components III (M-III) and also medium M-IV. The highest enzyme production was observed in the following order; sugarcane bagasse > wheat straw > rice husk, wheat bran > soybean meal > corncobs. The crude CMCase enzyme was applied for saccharification of agricultural wastes and maximum saccharification (11.45%) was observed in bagasse. These results suggested its potential utilization in particular biofuel industry.

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INTRODUCTION

The most abundant and renewable biomass on earth is cellulose which can be hydrolysed into soluble sugars by action of cellulases produced by microbes. Cellulases are enzymes which are synthesized by microorganisms when grown on cellulosic material (Lee and Koo, 2001). Cellulases can be produced by bacteria and fungi but fungi are the most potent producers. Among bacteria *Bacillus* sp. has ability to produce enzymes of industrial importance including cellulases (Priest, 1977).

Carboxymethyl cellulase (also known as endo-β-1,4-glucanase, EC 3.2.1.4) is one of the component of cellulase enzyme system that breakdowns 1,4-β-D-glucosidic bonds in the cellulose molecules (Siddiqui *et al.,* 1997). Carboxymethyl cellulase (CMCase) activity has been found in a variety of bacteria such as *Paenibacillus* (Pooyan and Shamalnasab, 2007), *Sinorhizobium fredii* (Chen *et al.,* 2004), *Bacillus cereus* (Yopi *et al.,* 2016), *Myxobacter* (Pedraza-Reyes and Gutierrez-Corona 1997), *Bacillus circulans* (Kim, 1995), *Cellulomonas flavigena* (Sami and Akhtar 1993), *Bacillus megaterium* (Shahid *et al.,* 2016), *Rhizobium leguminosarum* (Mateos *et al.,* 1992),

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Clostridium thermocellum (Reynolds *et al.,* 1986; Kobayashi *et al.,* 1990), *Thermonospora fusca* (Calza *et al.,* 1985) and *Cellulomonas uda* (Nakamura *et al.,* 1983).

In cellulase production selection of substrate is very important which can affect the cost of enzyme production. Mostly fungi have slow growth rate as compared to bacteria, so that's why bacterial strains are used for cellulase production. Bacterial cellulases are not commonly used in industrial processes due to lack of one of the three cellulase activities that is FPase. The greatest importance of the bacteria is that it could be genetically altered in order to enhance cellulase production (Ariffin *et al.,* 2006). For high yields of extracellular accumulation of cellulase it is necessary to develop microbial strains, media composition and process optimization (Gosh, 1987). The main objective of this study was to evaluate the various agricultural wastes and media optimization for maximum yield of CMCase enzyme from *Bacillus subtilis*-BS06 in submerged fermentation.

MATERIALS AND METHODS

Procurement of Substrates

Different agricultural wastes like sugarcane bagasse, wheat bran, rice husk, soybean meal, corn cobs and wheat straw were procured from market of local city Lahore and were used as substrates for CMCase enzyme production in submerged fermentation.

Bacterial Strain

Bacterial strain of *Bacillus subtilis*-BS06 was taken from Fermentation Laboratory, Food and Biotechnology Research Center (FBRC), PCSIR Laboratories complex, Ferozpure road Lahore, Pakistan. The strain was revived on nutrient agar (Oxoid) slants and store at 4^oC.

Cultivation of Vegetative cells

Twenty five milliliter of sterilized nutrient broth (Oxoid) was taken in 250ml Erlenmeyer flask and inoculated with a loopful of 24h old *Bacillus subtilis*-BS06 and kept in incubator at 37^oC for 24h with shaking speed of 140 rpm. These vegetative cells were used as a source of inoculum throughout the study.

Fermentation Media

In the present study four different types of media were used to check the CMCase production by *Bacillus subtilis*-BS06 under submerged fermentation. The compositions of the media used are given in Table I.

Table I: Composition (g/L) of different media used in this study.

Ingredient	Medium I	Medium II	Medium III	Medium IV
Yeast Extract	10	2.0	-	0.5
Peptone	20	-	-	-
KH_2PO_4	1.0	1.6	1.0	-
$Na_2HPO_4.12H_2O$	2.5	-	-	-
$MgSO_4.7H_2O$	0.32	5.0	0.16	0.16
$CaCl_2.H_2O$	-	-	0.08	-
K_2HPO_4	-	-	1.76	0.5
$(NH_4)_2SO_4$	-	-	5.0	-
Sucrose	-	-	-	20
NaCl	-	-	-	0.2

Fermentation technique

Twenty five milliliter of fermentation medium with 2% substrates were taken in 250ml Erlenmeyer flask and sterilized at 121^oC for 15min. The sterilized medium was inoculated with 2% solution containing vegetative cells of 24h old *Bacillus subtilis*-BS06 and incubated at 37^oC for different time periods with shaking speed of 140 rpm.

Preparation of enzyme

After the completion of the fermentation, the culture broth was filtered through muslin cloth and finally centrifuged at 4^oC, 8000 xg for

10 min to get clear liquid. The clear filtrate obtained was used as a source of crude enzyme.

Assay of CMCase enzyme

CMCase enzyme in the culture filtrate was estimated as reported earlier (Shahid *et al.,* 2016). Reaction mixture containing 500 µL of crude enzyme and 500 µL of 1% (w/v) CMC in 50 mM acetate buffer pH 5 was incubated at 50°C, for 30 min. The reaction was stopped by the addition of 1.5mL of DNS and boiled for 5 min. and absorbance was taken spectrophotometrically at 550nm. The sugars liberated were then measured with DNS (Miller, 1959). One unit enzyme activity was defined as the amount of enzyme required to produce 1 micro mole reducing sugar equivalent per minute under assay conditions.

Saccharification

In 500 ml Erlenmeyer flasks, 50ml of crude enzyme was taken and 1g of agricultural wastes were added and incubated for 8 hrs. at 50°C with agitation speed of 150 rpm. The liquid was centrifuged at 10,000 xg for 10 min. to get clear filtrate. Samples were taken to determine reducing and total sugars. The saccharification (%) was determined by following formulae (Irfan *et al.,* 2016).

Saccharification (%) =

$$\frac{Reducingsugarsreleased\ (\frac{mg}{ml})}{Substrateused\ (\frac{mg}{ml})} X\ 100$$

Statistical analysis

The data collected from experimentation was evaluated statistically by analysis of variance with significance level of p<0.05 using Microsoft Excel program. Each experiment was conducted in triplicate.

RESULTS AND DISCUSSION

Present study described the production of enzyme CMCase from bacterial strain of *Bacillus subtilis*-BS06 in submerged fermentation using various agricultural wastes as substrates. In this study different fermentation media were optimized under static and agitated conditions. For enhancement of yield medium, formulation is the prime step for successful laboratory experiments.

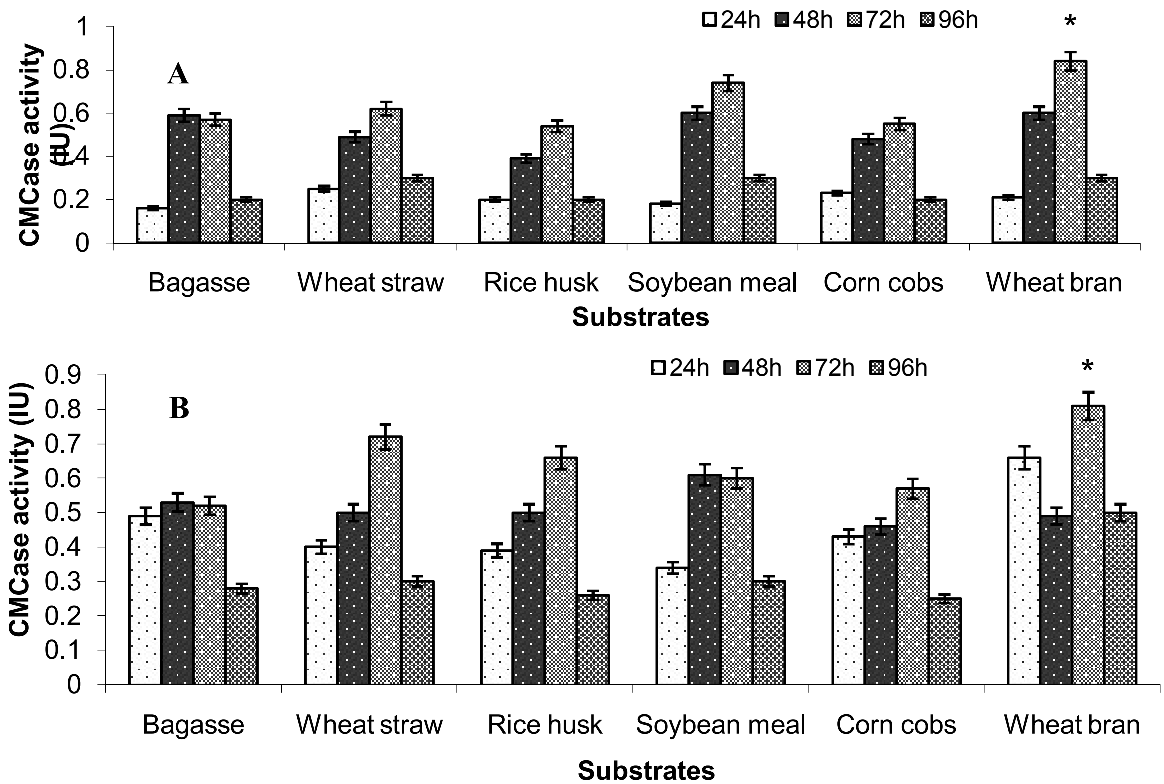

Figure 1. Production of CMCase by *Bacillus subtilis*-BS06 using medium-1 at 37°C (A) with agitation speed of 140rpm and (B) under static conditions. Bars represent the standard deviation among replicates and steric indicatesignificant difference at p<0.05.

Figure 1 showed the effect of Medium I (M-I) on CMCase production from *Bacillus subtilis*- BS06 using different substrates with agitation speed of 140 rpm up to 72h of fermentation period at 37°C. Results indicated that 72h of fermentation period was found optimum for CMCase production using wheat bran (0.84 ± 0.021 IU) as a substrate for submerged fermentation at 37°C with agitation speed of 140rpm. Lowest yield of enzyme production was observed at 24h and 48h of fermentation period with substrates other than wheat bran. When the same experiment was performed under static conditions (Fig. 1) same results (wheat bran, 72h) were found but the 24h and 48h of fermentation period also showed comparable results to 72h of fermentation period in different substrates other than wheat bran. So, by using medium component I wheat bran with 72h of fermentation period gave better yield in both static and agitated conditions. Otajevwo (2011) isolated many cellulolytic bacterial strains from rumen fluid, cow dung and soil, *Bacillus subtilis* is one of them which have ability to produce cellulase enzyme.

Heck *et al.* (2002) isolated strain of *Bacillus subtilis* from soil and water which has ability to secrete cellulase enzyme after 24h of fermentation of soybean industrial waste in solid state fermentation. Shabeb *et al.* (2010) produced cellulase from *Bacillus subtilis* KO using low cast medium and obtained maximum yield after 24h of fermentation using molasses as a carbon source. Selvankumar *et al.* (2011) produced endoglucanase from *Bacillus amyloliquifaciens* using coffee pulp as a substrate in solid state fermentation and obtained maximum yield of enzyme after 72h of incubation period.

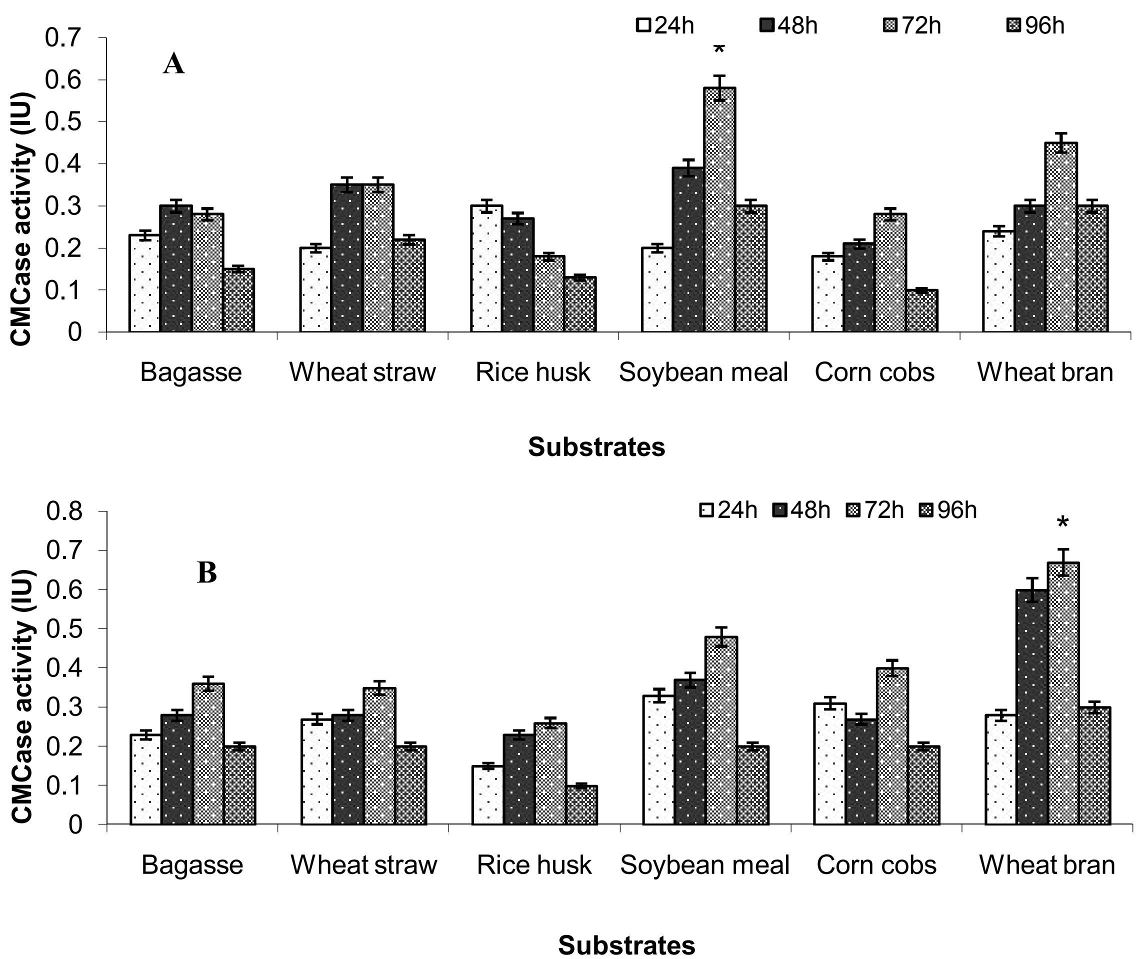

Figure 2. Production of CMCase by *Bacillus subtilis*-BS06 using medium-1I at 37°C (a) with agitation speed of 140rpm and (b) under static condition. Bars represent the standard deviation among replicates and steric indicates significant difference at p<0.05.

Odeniyi *et al.* (2009) isolated strain of *Bacillus coagulans* from palm fruit husk and tested for carboxymethylcellulase production. The best production was obtained after 72h of fermentation period under agitation conditions. Shafique *et al.* (2004) used strain of *Bacillus subtilis* for endoglucanase production in solid state fermentation of banana stalk and reported optimum production after 72h of fermentation without agitation. A strain of *Bacillus pumilus*EWBCM1 was isolated from the earthworm gut which has potential for secreting cellulase activities after 72h of fermentation period (Shankar and Isaiarasu, 2011).

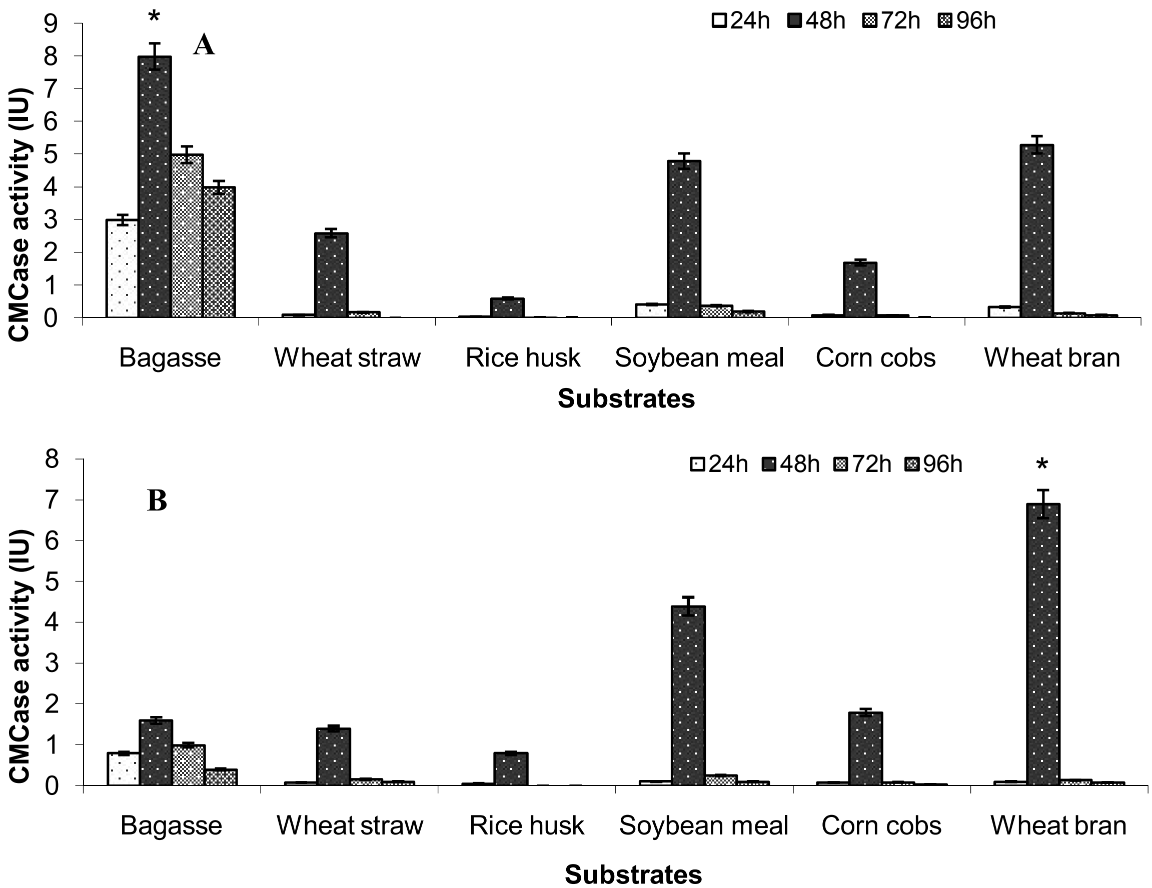

Figure 3. Production of CMCase by *Bacillus subtilis*-BS06 using medium-III at 37^{o}C (a) with agitation speed of 140rpm and (b) under static condition. Bars represent the standard deviation among replicates and steric indicates significant difference at p<0.05.

When medium components II were tested (Figure 2) soybean meal (0.58 ± 0.03 IU) gave highest CMCase activities after 72h of fermentation period with agitation speed of 140 rpm. On the other wheat bran (0.68 ± 0.021 IU) gave highest levels of CMCase production under static conditions after 72h of fermentation period. By changing substrate and fermentation period a great variation in enzyme production was observed. Ray *et al.,* (2007) isolated two bacterial strains *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 from fish gut which have great potential to produce cellulase enzyme and their optimum fermentation period was 96h in solid state fermentation. Figure 3 depicts the effect of medium constituents III with various substrates on CMCase production at 37^{o}C under static and agitated conditions. In this experiment, optimum fermentation period was found 48h with bagasse (agitation) and wheat bran (static) producing cellulase activities of 8.0 ± 0.27 IU and 6.9 ± 0.31 IU respectively. Fermentation periods of 24h and 72h did not show significant cellulose activities. Rice husk gave very little amount of enzyme production in both static and agitated conditions. Medium constituents III gave better yield as compared to media I & II. Majeed *et al.* (2016) reported optimized production of exoglucanase through response surface methodology using sugarcane bagasse as substrate by *Aeromonas besterium* at 35^{o}C for 24h of fermentation. Zambare and Christopher (2011) produced cellulase from *Bacillus amyloliquifaciens* UNPDV-22 and reported that wheat bran and soybean meal are the best sources for enzyme production. Figure 4 indicated the CMCase enzyme production using medium constituent IV (M-IV) with various agricultural wastes in agitated and static conditions at 37^{o}C. In this experiment three substrates i.e. sugarcane bagasse, wheat straw and rice husk produced better levels of cellulose enzyme production in both agitation and static conditions. Soybean meal, corn cobs and wheat bran showed no significant production with this medium supplementation. Sugarcane bagasse gave better enzyme production in both agitation after 48h (8.0 ± 0.32 IU) of fermentation and 72h (8.08 ± 0.25 IU) under static conditions. In static conditions, 72h of fermentation period was dominating while 24h eliminates the enzyme production. Under agitated conditions (bagasse, wheat straw & rice husk) 48h and 72h of fermentation period produce almost equal titers of cellulase enzyme in submerged fermentation at 37^{o}C.*Rhizobium* sp. DASA23010, *Escherichia coli* ATCC 25922 and *Bacillus subtilis* ATCC 6633 were examined for carboxymethyl cellulase production and it was observed that all these strains grow well on medium containing CMC as a carbon source and produce significant amount of cellulase enzyme (Punyathiti and Pongsilp, 2008). Nutrient supplementation to the medium greatly affects the enzyme production. Virupakshi *et al.,* (2005) observed maximum enzyme production by *Bacillus* sp. on rice bran medium with 72h of fermentation period. Kumar *et al.* (2009) isolated two strains of *Bacillus* sp. from flour mill waste. Among the two isolated strains, *Bacillus* sp. FME 2 has shown higher production of CMCase (100 U/ml), FPase (45U/ml) and ß-glucosidase (3.5U/ml) using rice husk as substrate for eight days of submerged fermentation. Various substrates like sugarcane bagasse (Khalid *et al.,* 2017), acacia sawdust (Anjum *et al.,* 2017), peanut shells (Arshad *et al.,* 2017), potato peels (Irfan *et al.,* 2017), banana peduncle (Arooj *et al.,* 2017) and eucalyptus leaves (Iqbal *et al.,* 2017) have been reported for cellulase production by *Bacillus subtilis* K-18 in submerged fermentation for 24h.

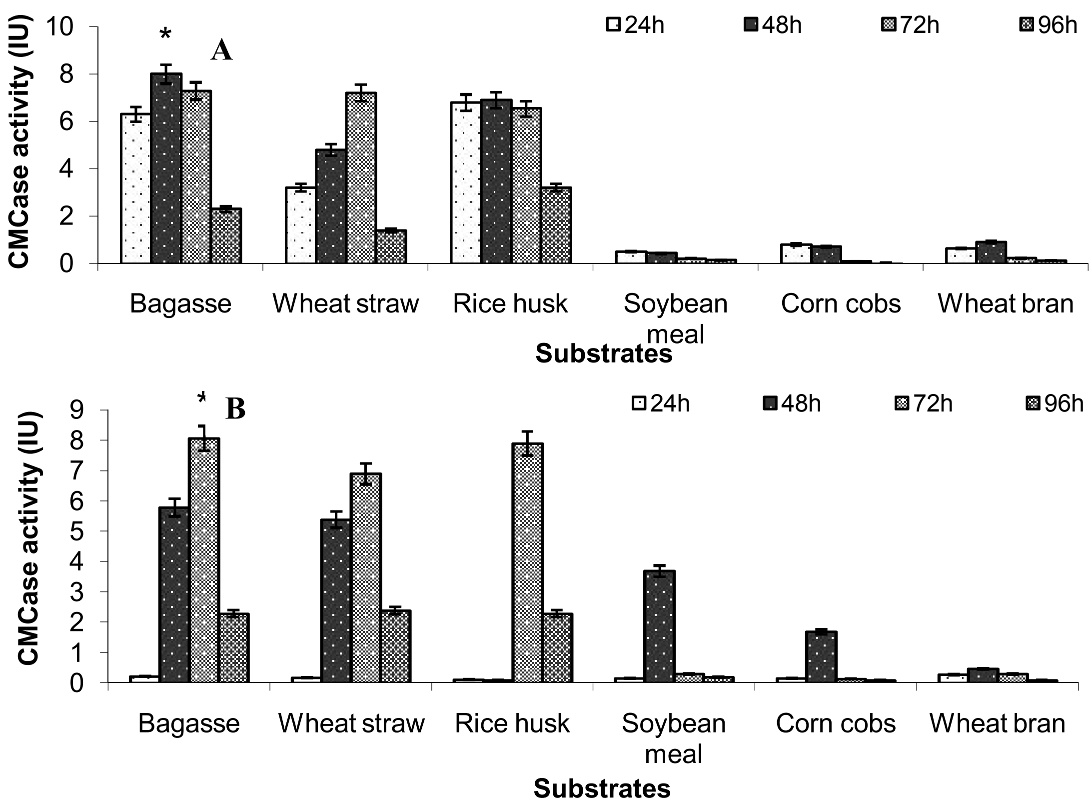

Figure 4. Production of CMCase by *Bacillus subtilis*-BS06 using medium-1V at 37^{o}C (a) with agitation speed of 140rpm and (b) under static condition. Bars represent the standard deviation among replicates and steric indicates significant difference at p<0.05.

The CMCase enzyme produced was applied for hydrolysis of these substrates for maximum liberation of sugars. Results (Fig. 5) showed that sugarcane bagasse released maximum sugars (2.29 ± 0.01 mg/ml) followed by wheat straw (1.47± 0.01 mg/ml) while rice husk produced less sugars (0.17 ± 0.01 mg/ml). The maximum sugars produced by sugarcane bagasse and wheat straw might be due to the higher percentages of cellulose and hemicellulose as compared to other agri-wastes. Kazeem *et al.* (2016) also reported that sugarcane bagasse as best substrate for cellulase production under shaking conditions with 180 rpm and the crude enzyme effectively hydrolyze sugarcane bagasse yielding reducing sugars of 0.348 g/g of dry substrate. The CMCase produced by *Bacillus licheniformis* AMF-07 yielded 32 and 24g/L reducing sugars from saccharification of wheat bran and rice straw respectively (Azadian *et al.,* 2016). Another study suggested that bacterial strains isolated from agricultural wastes had potential to produce hydrolytic enzymes which hydrolyze bagasse, potato peel, rice straw, saw dust and wheat straw (Abo-State *et al.,* 2016.

Conclusion

The results showed that maximum CMCase activity (8.0 ± 0.32 IU) was obtained by sugarcane bagasse as substrate after 48h of fermentation period under shaking conditions of 140 rpm at 37°C. The crude cellulase enzyme was applied for saccharification of lignocellulosic biomass yielding highest reducing sugars from sugarcane bagasse. These findings revealed that selection of suitable medium and fermentation conditions played a vital role in CMCase production by *Bacillus subtilis*-BS06 and are being considered as pre-requisites to make the process of enzyme production cost effective at large scale.

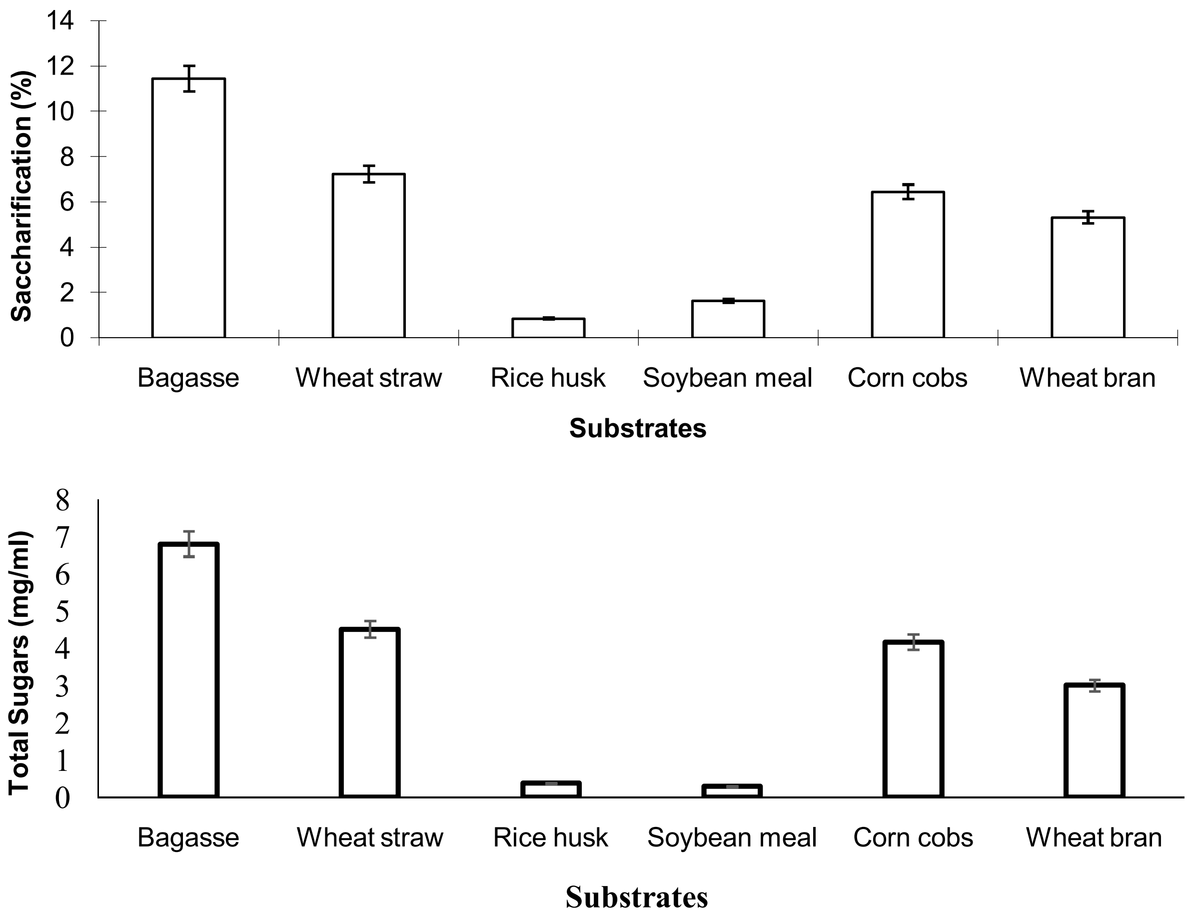

Figure 5. Saccharification (%) and total sugars released from agricultural wastes after 8h of incubation at 50°C.

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