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Optimizing Cellulase Production from Municipal Solid Waste (MSW) using Solid State Fermentation (SSF)

Jwan J. Abdullah[1,2*], Darren Greetham[1], Nattha Pensupa[1], Gregory A. Tucker[1] and Chenyu Du[1,3]

[1]Department of Bioenergy, University of Nottingham, UK
[2]Department of Environment, Salahaddin University-Erbil, Iraq
[3]Department of Environment, University of Huddersfield, UK

Abstract

This paper explores the possibility of using an industrially processed municipal solid waste (MSW) for cellulase enzyme production via solid state fermentation (SSF) by *Trichoderma reesei* and *Aspergillus niger*. Both fungi grew well on the MSW substrate and production of cellulase enzymes was optimized for temperature, moisture content, inoculation and period of incubation. The effect of additional minerals, and alternative carbon and nitrogen sources were also examined.

Following optimization a cellulase activity of 26.10 ± 3.09 FPU/g could be produced using *T. reesei* at 30˚C with a moisture content of 60% with an inoculums of 0.5 million spores/g and incubation for 168 hours. Addition of extra nitrogen and/or carbon did not improve cellulase accumulation. Acid or alkali pretreatment of MSW led to reduced cellulase production. Crude enzymes produced from MSW by *T. reesei* were evaluated for their ability to release glucose from MSW. A cellulose hydrolysis yield of 24.7% was achieved, which was close to that obtained using a commercial enzyme. Results demonstrated that MSW can be used as an inexpensive lignocellulosic material for the production of cellulase enzymes.

Keywords: Cellulase; Solid State Fermentation (SSF); Municipal Solid Waste (MSW); *Trichoderma reesei; Aspergillus niger;* Composition characterization

Introduction

Municipal solid waste (MSW) management is one of the key topics in environmental protection [1]. In England, around 23.7 million tons of household waste was generated in 2009/10, which equates to 1036 Kg per household [2]. The current technologies for MSW treatment are incineration, landfill, composting and anaerobic digestion. Recently, anaerobic conversion of MSW into biogas has attracted growing interest as a promising method to reduce environmental impact and to generate renewable fuel at the same time. However, some MSW contain over 50% lignocellulosic content [3] and anaerobic digestion may not represent the most efficient process. One alternatively approach is to hydrolyze the lignocellulosic component in the MSW into simple sugars and then ferment these sugars into ethanol. It was estimated that around 152 L of ethanol could be generated from a ton of processed MSW [3].

This enzymatic hydrolysis of lignocellulose to sugars requires a cocktail of enzymes that include mainly cellulase. Cellulase can be produced by various bacteria such as *Bacillus subtilus*, *Bacillus circulans* [4] (*Bacillus sphaericus-JS1* [5], *Cellulomonas flavigena* [6] and fungi like *Aspergillus sp*. [7] and *Trichoderma reesei* [8]. Among these cellulase producers, *Trichoderma reesei* and *Aspergillus niger* have attracted most attention due to their high cellulase productivity, safe use in industry and the availability of their whole genome sequences. Both submerged fermentation and solid state fermentation (SSF) have been used in cellulase production [9]. In comparison with submerged fermentation, SSF is easy to operate, requires minimum equipment and has the unique advantage of being able to handle insoluble solid substrates, such as lignocellulose. Therefore, it has been widely used in the fermentation of agricultural residues and food processing wastes. Various substrates, such as wheat straw, sugar cane bagasse and oil palm biomass have been examined for cellulase production using *T. reesei* and *A. niger*.

Research has revealed that *T. reesei* can produce 13.4 FPU/g cellulase activity using water hyacinth [8]; 154.58 FPU/g using sugar cane bagasse [10]; 8.2 FPU/g using oil palm empty fruit bunches [11]; and 1.16 FPU/g using rice bran [12] as substrates. Similarly it has been reported that *A. Niger* could produce 24 FPU/g cellulase activity using wheat straw as a substrate [13]. Other substrates such as banana peel, rice straw, corn cob residue, rice husk, banana fruit stalk, and coconut coir pith have all being used for cellulase production [14,15].

Although MSW can contain a high lignocellulosic content, there has been limited research into the use of MSW for cellulase production. Other research demonstrated the possibility of using MSW to produce cellulase via SSF. But by using raw not autoclave MSW [16,17]. In this paper, we report an example of using an industrially processed MSW for cellulase production and then the use of the resulting "cellulase cocktail" for the enzymatic hydrolysis of MSW.

Materials and Methods

Municipal solid waste

Municipal solid waste (MSW) was kindly supplied by Wilson Steam Storage Ltd (UK). The MSW had been subjected to commercial steam

***Corresponding author:** Jwan J. Abdullah, Department of Bioenergy, University of Nottingham, School of Biosciences, Sutton Bonington Campus, Loughborough, LE12 5RD, UK, Tel: 07761817046; E-mail: jwan4@outlook.com

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autoclaving at 165°C to generate an organic rich fraction. The MSW as received was sieved using a 2 mm sieve, and the fraction that passed through the sieve was collected and dried in an oven at 70°C overnight.

MSW characterization

Cellulose and hemicellulose: Cellulose and hemicellulose were analyzed using acid hydrolysis according to the methods described by the National Renewable Energy Laboratory (NREL/TP-500-42618). A 30 mg sample of MSW was added to 1 mL of 12 M H_2SO_4 incubated at 37°C for 1 hour then 11 mL of distilled water was added and the samples was placed in a water bath at 98°C for 2 hours [18]. Then the hydrolyzed simple sugars were determined using a HPLC (Dionex). Monosaccharides (arabinose, galactose, glucose and xylose) were analyzed using Dionex ICS-3000 Reagent-FreeTM Ion Chromatography equipped with Dionex ICS-3000 system, electrochemical detection using ED 40 and computer controller. A CarboPacTM PA 20 column (3 x 150 mm) was used and the mobile phase was 10 mM NaOH with a flow rate of 0.5 mL/min. The injection volume was 10 µL and the column temperature was 30°C.

Lignin: Lignin was determined using the acetyl bromide method as described by Sluiter et al. [19]. A 100 mg sample of MSW was added to 4 mL 25% acetyl bromide in glacial acetic acid and incubated in a water bath (50°C) for 2 hours. The tubes were cooled and a further 12 mL of glacial acetic acid added. Tubes were then centrifuged at 3000 rpm (1609 g) for 5 min. 0.5 mL supernatant was transferred to a new falcon tube and 2.5 mL of glacial acetic acid, 1.5 mL of 0.3 M NaOH, 0.5 mL (0.5 M) hydroxylamine hydrochloride added. Finally glacial acetic acid was added to make a final volume of 10 mL, absorbance was measured at 280 nm and the concentration of lignin calculated by comparison with standards containing 0.4, 0.6, 0.8, 1.0 and 1.2 mg lignin (Sigma Aldrich) [19].

Total nitrogen and protein: Total nitrogen and protein were determined using a Nitrogen/Protein Analyzer (Thermo Scientific Flash EA1112) with L-aspartic acid as the standard and the method as described by Campbell et al. [20].

Total lipid analysis: The lipid content was measured using the Folch method as described by Cequier-Sánchez et al. [21]. MSW sample (400 mg) sample of was added to 12 mL of dichloromethane/methanol (2: 1, v/v) and incubated for 2 hours at room temperature with occasional mixing. Following incubation samples were centrifuged at 1000 rpm (178 g) for 5 mins and the upper organic phase was carefully removed using a glass syringe and transferred into a clean 50 mL glass centrifuge tube. 2.5 mL KCl (0.88%, w/v) was added and the sample was centrifuged at 1000 rpm (178 g) for 5 mins and the lower organic phase was carefully transferred into a pre-weighed glass tube. The lower organic phase was dried under nitrogen gas until all liquid had evaporated. The tube was reweighed to give the total lipid content [21].

Trace element analysis: 2 g sample of MSW was added to 15 mL of concentrated HNO_3 and placed on a hot plate until the volume reached 5 mL. Samples were then filtered using filter paper (No. 42) and distilled water was added to make a final volume of 100 mL. The samples and blank (no MSW) were then analyzed using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Major elements (Ca, Mg, K, and Na) were analyzed at the ppm (mg/L) level with the detector operating in analogue mode only. While for the minor elements data were expressed as mg/Kg [22].

Acid and alkali pre-treatments: MSW (2 g) was added to 100 mL of either 1% H_2SO_4 or 2% NaOH and autoclaved at 121°C for 30 min or 15 min, respectively. After autoclaving, samples were neutralized to pH 7.0 using either 1 M NaOH or 1 M H_2SO_4 as appropriate. The samples were then centrifuged at 5000 rpm (4472 g) for 10 min. The solid fraction (pellet) was rinsed three times by adding distilled water to the pellet and re-centrifuged. The solid fraction was then dried overnight at room temperature.

Microorganisms: Two filamentous fungi, *Trichoderma reesei* QM6a and *Aspergillus niger* N402 were kindly donated by Professor David Archer (University of Nottingham, UK). Procedures for storing and cultivating *T. reesei and A. niger* were as previously described by Ries et al. [23].

Solid State Fermentation (SSF): A 6 g (dry weight) of MSW was placed into a 250 mL Duran bottle. Sufficient distilled water was then added to the MSW to give a final moisture content of 60, 70 or 80%. The Duran bottles were autoclaved at 121°C for 15 min. After sterilization the substrate was cooled down to room temperature. Spore suspensions of *T. reesei* or *A. niger* (0.5, 1 or 2×10^6 spores/g of dry MSW) were then added. The mash was mixed using a sterilized spatula and approximately 2.0 g of inoculated MSW mash was distributed to Petri dishes. The Petri dishes were then incubated at 25, 28 or 30°C using a static incubator for up to 168 hours.

Addition of mineral solutions, carbon source, nitrogen source and clay: With the aim of improving cellulose production, the addition of mineral solutions, carbon source, nitrogen source and clay was carried out. Two stock solutions of trace metals were prepared; mineral solution 1 contains 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$ in 1000 mL distilled water and mineral solution 2 contains 26 g KCl, 26 g $MgSO_4$,76 g KH_2PO_4 in 1000 mL of distilled water. In the experiments of using a mineral solution, the mineral solution was used to adjust moisture content instead of distilled water. In the experiments investigating the impact of various nutrient/chemical reagent addition, 0.2% or 1% (w/w) cellulose powder, peptone, clay or combined cellulose powder and peptone was added into MSW, as the carbon source, nitrogen source, reagent to adsorb toxic chemicals and combined carbon and nitrogen source, respectively. The addition was mixed with MSW before autoclave.

Extraction and assay of cellulase activity: The method used to extract cellulase activity was as described previously by Pensupa et al. [13]. Fermented solid samples from Petri dishes containing 2.0 g of original dry substrate were mixed with 16 mL of 0.05 M sodium citrate buffer (pH 4.8) and blended for 10 seconds using a food processing blender. The suspension was then poured into a beaker and stirred at 300 rpm for 30 min at 4°C. Samples were centrifuged at 5000 rpm (4472 g) for 10 mins. The supernatant, containing crude enzymes, was retained and used for enzymatic activity analysis.

Cellulase activity was measured as filter paper units (FPU) following the protocol of Adney et al. [24]. Crude extract (0.5 mL) was mixed with 0.5 mL of 50 mM citrate buffer (pH 4.8). A strip (1 × 6 cm, approximately 50 mg) of Whatman No. 1 paper (cellulose substrate) was placed into the tube, and the tube was incubated at 50°C for 1hour. After that, 3 mL of dinitrosalicylic acid (DNS) reagent (prepared according to Adney et al. [24] was added to each tube and the mixture was boiled for 5 mins. After cooling on ice for 5 mins, samples were diluted (0.2 mL of sample mixed with 2.5 mL of distilled water) and absorption was read at 540 nm. One unit (FPU) of enzyme activity was defined as the amount of enzyme required to liberate 1 mmol of glucose per min.

Enzymatic hydrolysis: Fungal extract (8.3 mL) was mixed with 91.7 mL sodium citrate buffer solution (50 mM, pH 4.8) to achieve an

enzyme loading rate of around 30 FPU per g dry weight of MSW. A commercial cellulase Ctec2 (Novozyme) at the same enzyme loading was used as a control.

The hydrolysis was started by adding 0.5 g (dry weight) of substrate into the fungal extract or control enzyme solutions, and then the samples were shaken at 150 rpm in a shaking incubator at 50°C for 72 hours. Samples were taken at 0, 2, 4, 12, 24, 48 and 72 hours. Samples were centrifuged at 5000 rpm (4472 g) for 10 mins and the supernatant was analyzed for glucose concentration using a Dionex HPLC.

Transcriptome analysis of *T. reesei* during SSF on MSW: *T. reesei* was cultured on MSW incubated at 30°C under optimal conditions and fungal samples collected at three time points 96,120 and 168 hours and frozen until required. The mycelia were frozen using liquid N_2 and ground using a pestle and mortar. The ground mycelium was then gradually added to 1 mL of Trizol (invitrogen) in a 2 mL tube to give a final volume of around 1.5 mL. The samples were, left at room temperature for 10min. Then 200 µl of chloroform was added to each sample, which were then vortexed and left for 2-3 min. Samples were then vortex again, then centrifuged at 13000 rpm (37788.4 xg) for 10 min. 750 µl of the upper aqueous phase was removed and placed into a 1.5 mL tube. 750 µl of isopropanol was added and samples left for 20 min on ice to precipitate the RNA, Samples were centrifuged at 13000 rpm (37788.4 xg) for 10 min this resulted in a gel like pellet being formed on the side and bottom of the tube. The supernatant was removed and pellet washed with 700 µl of 70% ethanol, centrifuged at 13000 rpm (37788.4 xg) for 10 min. The ethanol was removed and the pellet left to dry in a laminar airflow cabinet for 10-15 mins. The pellet was resuspend in 100 µl of DEPC (Diethylpyrocarbonate) treated water. RNA clean up kit (Qiagen) was used to remove DNA following the manufacturers "on column DNase digest" protocol.

RNA samples were sent for quality control and RNAseq to the service centre. The University of Nottingham. Annotation of the reads and the initial global analyses was carried out by Dr. Martyn Blythe. Three biological replicates were prepared for each time point. Unfortunately, one of the three replicates from day 5 failed the internal RNAseq quality control and as such was not used in the subsequent analysis.

A Filtering Pipeline was used to filter reads with low sequencing score and reads aligned to adaptor sequences. Initially raw reads were trimmed for adapter sequences using Sythe. This was followed by quality trimming using Sickle. Reads that passed the filter were then aligned to the reference and reads were removed which mapped to the rRNA and tRNA genes (as annotated in the genome reference). Then reads that passed the rRNA and tRNA filter were mapped onto the reference genome in the context of known gene exon coordinates by tophat mapping tool. The reference genome used was *T. reesei* Version2 as provided by the Joint Genome Institute (JGI) through their WEB site. It was found however, that the reference genome had poor annotation.

Read counts for each gene were calculated using 'htseq-count'. This program determines the number of uniquely aligned reads per gene. MAPQ30 (Unique) was then used to correctly mapped reads and to generate counts per gene. Gene expression was expressed as Reads Per Kilobase of transcript per Million mapped reads (RPKM) values. The RPKM is simply a normalized read count (stranded/sense reads) for a given gene as defined by the University of California Santa Cruz (UCSC) ref Gene database. The read count of the exon-space of a gene is normalised against the total number of mapped reads (Uniquely and correctly mapped reads with rRNA excluded) in that particular alignment file, and against the total length of the gene's exon-space.

Statistical analysis

All experiments were carried out in triplicate. Microsoft Excel was used to calculate data means and standard deviations. ANOVA was performed using either Design Expert or SPSS.

Results and Discussion

Composition of MSW

The chemical composition of the MSW was assessed as described in the methods (Table 1). The results showed that the MSW contained significant levels of cellulose, hemicellulose and lignin. The total hydrocarbon content was over 60%, indicating that it was a carbon rich waste stream, which could be used as a potential substrate for biofuel production. The cellulose content of the MSW (27.8 g/100 g) was similar to that reported by Barlaz et al. and Jones et al. [25,26], which were 28.8 and 25.6 g/100 g, respectively. Some researchers however, have reported much higher cellulose in MSW derived principally from the paper, wood and milling industries [27,28].

The hemicellulose content (15.45 g/100 g) was again similar to the 11.9 g/100 g reported by Jones et al. [26]. However, these are higher than several other values for example 5.14, 5.8 and 6.6 g/100 g as reported by Ham et al., Price et al. and Barlaz [29-31], respectively. Lignin content of our MSW (17.7 g/100 g) was close to the values reported by Barlaz et al. and Ham et al. [28,29] these being 12.67; 15.7 and 15.2 g/100 g, respectively. However, some authors reported higher values of lignin for example 25.1 g/100 g [30].

The amount of lipid (11.2 g/100 g) of this MSW was similar to that reported for a MSW sample collected from a Mechanical-Biological Treatment (MBT) plant (Barcelona, Spain), which was 11.52 g/100 g [32]. Low lipid content (4.9 g/100 g) had been reported from a MSW sample derived from food wastes emanating from fruit and vegetable markets, households, hotels and juice production centers [33]. Generally, MSW that contains waste food materials have a higher lipid content than those which are derived from wood and paper [34,35]. The protein and total nitrogen concentrations of this MSW sample were similar to those reported by Ponsá et al. and Rao et al. [32,33]. A high proportion of the nitrogen present in the MSW was in the form of protein (5.9 g/100 g), indicating that the MSW could be a good nitrogen resource for microorganism fermentation in addition to carbon resource.

This variation in composition between the MSW used in this work and that reported by others is to be expected given the variable nature of how the MSW is collected and processed. However, it does demonstrate that he MSW used in this paper could be representative of a much wider range of waste material.

The trace element composition of the MSW is shown in Table 2. Of the major elements (Na, Mg, P, S, K, and Ca), Ca had the highest concentration at 25064.74 ± 1550 mg/Kg. Presence of high levels of calcium was probably due to the lime spray treatment during the sterilization process. Sulphur recorded as the second highest major element (5392.4 mg/Kg). Other elements present at more than 1000 mg/Kg were K, Mg, Na, Al, Fe, Cu and P. The only other elements found in significant quantities were Zn and Mn (Table 2).

Trace elements can be classified into three classes based on their biological function and effects: (1) the essential metals (Na, K, Mg, Ca, V, Mn, Fe, Co, Ni, Cu, Zn, Mo and W); (2) toxic metals (Ag, Cd, Sn, Au, Hg, Ti, Pb, Al, Ge, As, Sb, and Se and metalloids) and (3) non-essential,

Structural component	g/100g
Cellulose	27.8 ± 0.1
Hemicellulose	15.45 ± 0.07
Lignin	17.7 ± 0.05
Lipid	11.2 ± 0.1
Protein	5.9 ± 0.2

Table 1: Chemical composition of municipal solid waste. Mean ± SD (n = 3).

Metals	mg/Kg	Metals	mg/Kg
Na	2509.27 ± 145	**Ni**	93.47 ± 28
Mg	2722.43 ± 254	**Cu**	1844.95 ± 116
P	1113.66 ± 151	**Zn**	612.68 ± 43
S	5392.43 ± 299	**As**	2.98 ± 0.4
K	2190.69 ± 120	**Se**	0.13 ± 0.02
Ca	25064.74 ± 1550	**Rb**	2.32 ± 0.14
B	41.56 ± 4.8	**Sr**	63.41 ± 7
Ti	21.88 ± 1	**Mo**	3.39 ± 0.08
Al	3120.61 ± 119	**Ag**	3.15 ± 0.38
V	23.88 ± 0.9	**Cd**	33.92 ± 2
Cr	15.96 ± 0.7	**Cs**	0.15 ± 0.012
Mn	397.73 ± 3	**Ba**	291.22 ± 24
Fe	3851.89 ± 200	**Pb**	161.19 ± 9
Co	11.27 ± 0.6	**U**	0.23 ± 0.018

Table 2: Elements composition of autoclaved municipal solid waste. Mean ± SD (n = 3).

non-toxic metals (Rb, Cs, Sr and T) [36].

The MSW sample contained high concentrations of several of the essential elements and some of the toxic metals. The main sources of heavy metals in MSW are usually batteries (Ni, Zn, and Cd); due to the poor availability of recycling facilities for hazardous wastes and poor public attitudes to waste management [37,38].

In addition other materials such as paints, electronics, ceramics, plastics and inks/dyes can all contribute to the heavy metal burden of MSW [37,39]. Generally, paper fractions contain the highest concentration of these metals [40].

The levels of some of the major elements, such as Na and K are higher in this present study than in some previous reports [41]. However, some previously published data suggested that the K content in our study was actually lower [42]. This again reflects the wide variations expected in MSW and was possibly due to a higher concentration of salty food wastes and plastic materials in the MSW, however, the current results generally agree with those of Park et al. [42]. Similarly a comparison of the present study with others studies shows that the Ca content was almost identical to two previously published papers [43,44], but lower than other published papers [41,45].

Comparison of SSF of MSW and wheat straw

In a previous study, wheat straw had been successfully used for cellulase production in SSF using wheat straw as a substrate [13]. Considering the similarity of the lignocellulosic composition of this MSW sample to wheat straw (cellulose 31.7%, hemicellulose 17.9% and lignin 20.2%), in the first instance a comparison was made between MSW and wheat straw as the sole substrates, using the optimum conditions identified for wheat straw [13] and comparing *T. reesei* and *A. niger* (Figure 1). Our previous results demonstrated that 3 days of SSF was best for cellulase production using wheat straw [13] and this agrees with the result here where after 5 days cellulase production, especially with. *A. niger,* was reduced. At 3 days *A. niger* performed

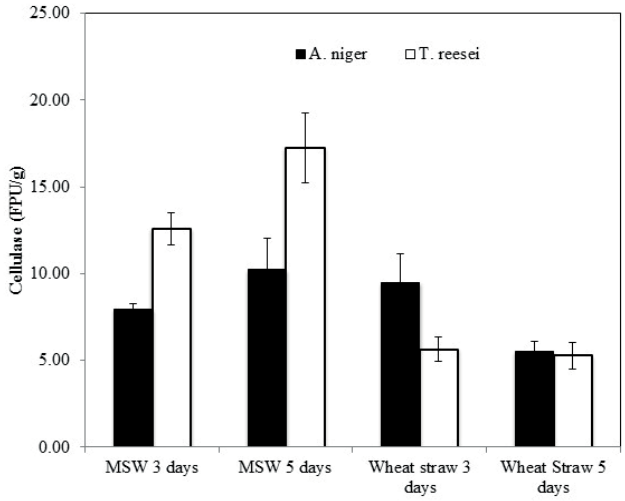

Figure 1: Comparison of cellulase production using municipal solid waste (MSW) and wheat straw (WS). Solid state fermentations of MSW were set up at 60% moisture content. These were inoculated with either *T. reesei* or *A. niger* at an inoculation size of 1 x 10^6 spores/g for either 3 days or 5 days. Results presented are the mean ± SD (n = 3).

better than *T. reesei* in term of cellulase formation using wheat straw as the substrate. In contrast Figure 1 clearly showed that extending culture time from 3 days to 5 days increased cellulase production in SSF using MSW, possibly indicating that fungi growing on MSW-based substrate required extra time to adapt to the growth environment. The increased fermentation time might be due to the heavy metal content of the MSW. It was also clear that, especially at day 5, *T. reesei* produced higher cellulase activity than *A. niger* when MSW was used as the substrate. A SSF time of 5 days was used as the standard for the following optimization experiments.

Optimization of moisture content and temperature

The combined impact of moisture content and temperature on cellulase production by both *T. reesei* and *A. niger* was investigated. A series of SSF were set up with moisture contents of 60, 70 or 80%, and fermentation temperatures of 25, 28 or 30°C. These were inoculated with either *T. reesei* or *A. niger* spores at a concentration of 1x10^6 spores/g and left to ferment for 120 h (5 days). Cellulase was extracted and assayed at the end of the fermentation and the results are shown in Figure 2. It can be seen that *T. reesei* produced significantly higher amounts of cellulase than *A. niger* under all conditions (p=0.0021). There was a statistically significant effect of moisture content (p=0.003) with 60% giving the highest enzyme activity, at all three temperatures, for both *T. reesei* and *A. niger*. There was also a significant effect of temperature (p=0.0092) on cellulase enzyme production. In this study, the best temperature for *T. reesei* was 30°C, whilst 25°C resulted in maximum production of cellulase by *A. niger.*

Moisture content is generally considered to be a crucial factor that affects oxygen transfer and nutrient accessibility in SSF. High moisture encourages fungal growth, nutrient transportation and enzyme activities, but limits oxygen transfer and facilitates contamination [46,47]. A wide range of moisture contents from 50-89.5% have been used in various studies for cellulase production using *A. niger* [13]. In the case of the MSW used in this study 60% was the minimal moisture content possible due to the water absorbing properties of the substrate.

In addition to the preference of the microorganism, optimum moisture content may also be influenced by properties of the substrate such as porosity and particle size [46,48].

Temperature is another important factor that affects fungal growth and enzyme production. SSF of *T. reesei* and *A. niger* is normally operated within the temperature range of 25 to 30°C [10]. Many studies have been carried out to optimize the incubation temperature for *T. reesei* and *A. niger*. The combined impact of temperature and moisture content has also been reported in similarly studies using spent brewing grains (SBG), rice bran and soybean hulls supplemented with wheat bran [12,49,50].

The highest cellulase activity obtained was 18.98 ± 0.65 FPU/g, at 60% moisture content, 30°C, in SSF using *T. reesei*. As *T. reesei* performed better in SSF of MSW, it was selected for further optimization experiments.

Optimization of inoculation size

Using the moisture and temperature condition determined above the effect of inoculum size on cellulase production by *T. reesei* was examined using spore suspension inoculations of 0.5×10^6, 1×10^6 and 2×10^6 spores/g dry weights MSW. The results are shown in Figure 3. The maximum cellulase activity (19.13 ± 1.5 FPU/g) was obtained in fermentations using 0.5×10^6 spores/g. Increasing the inoculation size from 0.5×10^6 to 1×10^6 and 2×10^6 spores/g, resulted in a significant decrease ($p = 0.0045$) in recovered cellulase activities to 17.17 ± 2.91 and 15.03 ± 2.81 FPU/g, respectively. Decreased cellulase productivity with an increased inoculation size could be explained by the resultant higher amounts of biomass depleting the nutrient pool and the available oxygen at the early stages of growth, which then affected the cellulase formation [51]. Alternately the decrease in cellulase production even after inoculating the media with higher spore concentrations might be due to the creation of anaerobic conditions or a nutritional imbalance as a result of the more rapid growth of the microorganisms [52,53].

Effect of additions of Minerals and supplements to MSW on cellulase production

Using the optimal conditions determined above the effect of adding additional mineral supplements, additional carbon and nitrogen sources or the inclusion of clay to absorb potentially toxic elements were examined. The addition of mineral solution one did not increase cellulase production, whilst addition of mineral solution two resulted in a significant reduction in enzyme activity ($p=0.0044$). The results for the addition of the other supplements are shown in Figure 4.

In the case of the other additions, additional carbon (cellulose powder), nitrogen (peptone) or the addition of clay did not result in any significant changes in cellulase production as compared with the control. These results might imply that the MSW has sufficient intrinsic nutrients and that additional supplementation is not required. The only significant differences observed in Figure 4 were between the cellulase production in the presence of 0.2% clay and with some of the fermentations with the addition of carbon and/or nitrogen. The addition of clay may result in the removal of inhibitory heavy metals and thus encourage fungal growth, there was a slight increase in cellulase production following the addition of 0.2% clay but this was not statistically significant. Similarly the addition of alternate carbon or nitrogen sources may act to deflect production of cellulase by the fungus. There was a general decrease in cellulase production observed with the addition of these nutrients but again this was not significant when compared to the control.

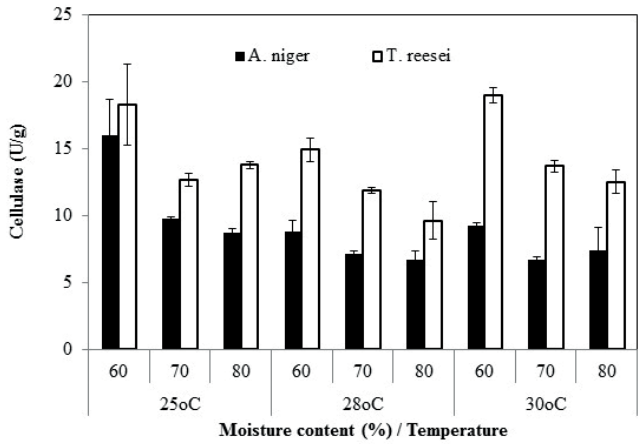

Figure 2: Impact of moisture and temperature on cellulase production. Solid state fermentations of MSW were set up at 60, 70 or 80% moisture content. These were inoculated with either *T. reesei* or *A. niger* at an inoculation size of 1×10^6 spores/g. Plates were then incubated at 25, 28 or 30°C for 120 hours and cellulase production determined. Results presented are the mean ± SD (n = 3).

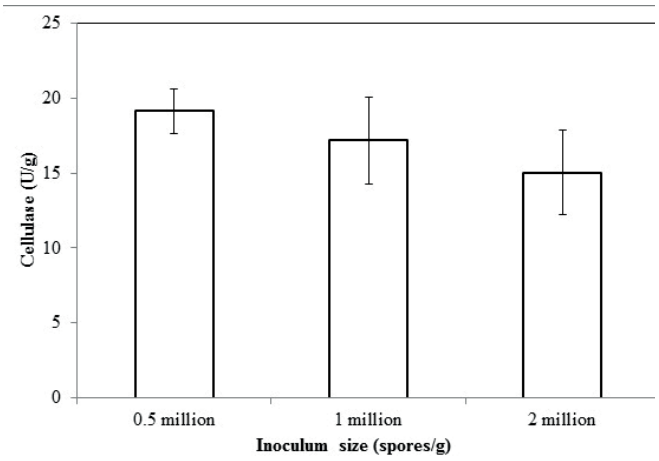

Figure 3: Impact of inoculum dose on cellulose production. SSF of MSW using *T. reesei* at three different inoculation doses were carried out using 60% moisture at 30°C. Crude enzyme was extracted after 120 hours and analyzed for cellulase activity. Results presented are the mean ± SD (n = 3).

Effect of pretreating MSW on cellulase production

The impact of pretreatments, designed to increase the accessibility of the cellulose on cellulase production was examined. The MSW was subjected to either an acid or alkali pretreatment prior to SSF. The results showed that in both cases pretreatment actually resulted in reduced cellulase production compared to non-treated MSW. Pretreatment with acid resulted in a significantly lower cellulase production of 47.8% of that obtained in the control fermentations, whilst that with alkali was only slightly higher (65.2%).

Modification of substrate is a common practice to increase the accessibility of the substrate to microorganisms [54,55]. In a previous study, it was found that acid modification of wheat straw significantly enhanced cellulase production in SFF by *A. niger* [13] v. However, a negative impact of pre-treatment on cellulase production has also

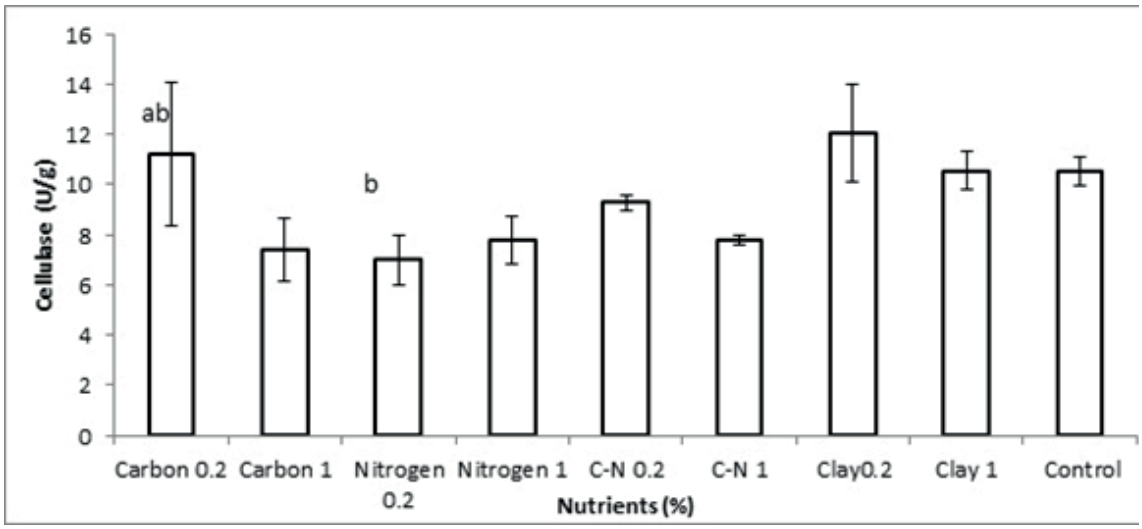

Figure 4: Impact of additional carbon, nitrogen or clay on cellulose production. SSF of MSW using *T. reesei,* at an inoculation size of 1 × 10^6 spores/g, were carried out using 60% moisture at 30˚C. The effect of adding additional carbon (cellulose powder) or nitrogen (peptone) nutrient sources, either individually or in combination was examined. The addition of clay at either 0.2 or 1 % (w/w) was also tested. All fermentations were compared to a control. Crude enzyme was extracted after 120 hours and analysed for cellulase activity. Results presented are the mean ± SD (n = 3). Bars with the same letters indicate no significant difference ($p > 0.05$).

been reported by several researchers [56-58]. The decrease in cellulase production could be attributed to (1) structural changes to the lignocellulosic raw materials caused by the alkali pretreatment [56,59]; (2) the generation of inhibitory compounds formed during the acid or alkali pretreatments [56]; (3) the removal of certain nutrients, such as nitrogen by the pretreatment [57,58] and (4) the release of toxic heavy metals that were originally insoluble in the MSW.

Cellulase production under optimal conditions

The optimization of conditions for cellulase production described above utilized a fixed SSF incubation of 120 hours. The effect of extending this culture time from 5 days to 7 days was thus examined. This extended fermentation of cellulase production from 18.53 ± 0.19 to 26.10 ± 3.09 FPU/g. Longer fermentation times were not explored as they were felt to be commercially insignificant. The length of incubation period is a prime concern for the development of a commercial cellulase production process and 7 days may not be viable. Various studies showed that maximum cellulase production from *T. reesei* could be achieved within 72-96 hours, the optimal was at 72 hours using cassava bagasse, wheat bran or rice straw [10]. Also the maximum cellulase produced using apple pomace (2.3 FPU/g) was after 120 hours [60]. The elongated cellulase production period maybe due to the characteristics of this MSW substrate, which contained toxic compounds and substrates from various carbon resources. Compared with homogeneous substrates, fungal cells required a longer time to grow and to express cellulase enzymes.

Hydrolysis of MSW using crude fungal extract

The crude fungal extract obtained from an optimum fermentation was collected. This was then used for the enzymatic hydrolysis of a fresh MSW sample with a cellulase enzyme loading rate of 30 FPU/g dry weights. A commercial cellulase cocktail, Ctec2 (Novozymes), was used as a control at the same enzyme loading rate. Glucose release with time for the hydrolysis is shown in Figure 5. The glucose concentration in the hydrolysis using Ctec2 reached 90% of its final concentration with 24

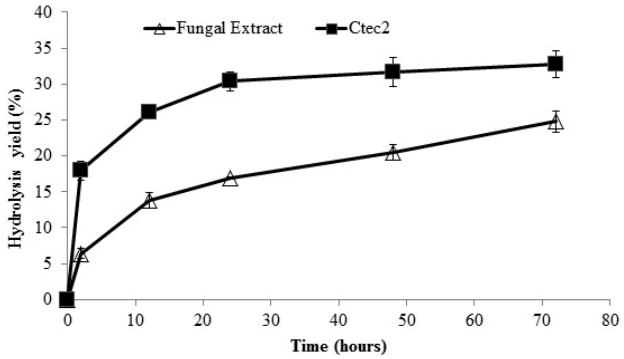

Figure 5: Glucose release during the hydrolysis of MSW using either commercial or MSW derived cellulase cocktails. Untreated MSW was incubated with either a commercial enzyme (Ctec2) or the crude enzyme as obtained from SSF of MSW (MSW). In each case at an enzyme loading rate of 30 FPU/g dry weight. Results presented are the mean ± SD (n = 3).

hours. This represents a 32.8% yield of available glucose in the MSW. In the hydrolysis using the fungal extract from the SSF, the initial glucose release rate was slower compared to that with Ctec2 and the final yield was around 24.7%. Although lower than the commercial enzyme, the hydrolysis achievable with the cellulase extract from *T. reesei* grown on MSW was nonetheless significant.

Transcriptome analysis

Using *T. reesei*, SSF was carried out under optimal conditions and fungal mycelium harvested at 3, 5 and 7 days. RNA was extracted from the mycelium and sent to the service centre at The University of Nottingham for RNAseq analysis. Initial filtering and mapping of the data was carried out by the service centre. Unfortunately one of the three replicates for day 5 failed the quality control and as such has been removed from this analysis. Figure 6 shows the results of the trimming and mapping exercises as carried out by the bioinformatics service. S1-3 represent the triplicate samples from day 3, S4 and 6 the

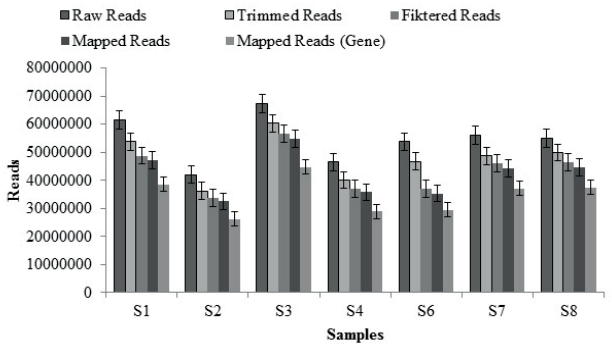

Figure 6: Filtering and mapping data for individual samples. Quality control and initial filtering and mapping were carried out by the service centre as described in the method.

samples from day 5 and S7-9 the triplicate samples from day 7. S5 is missing as this failed the quality control. S9 data was not provided by the bioinformatics centre raw counts ranged from 40 to 68 million and in all cases the filtering and mapping to genes has reduced the number. However, there were still 20 to 38 million reads that could be mapped to genes and a total of 9, 143 individual genes were finally identified and quantified.

PCA analysis of the mapped genes from the individual samples showed three clear clusters of samples 1, 2 and 3; 4 and 6 and 7, 8 and 9. Indicating that there was good replication between the biological replicates and that overall gene expression on the three sampling days was significantly different (data not shown).

The RPKM values for all 8 samples was transferred to an excel spread sheet that has been generated by Dr. Paul Daly (The University of Nottingham) for further analysis.

The spread sheet was designed to specifically analyse those carbohydrate active enzymes that are listed on the CaZy website. Thus the preliminary analysis of gene expression on the three days of sampling was restricted to this class of enzymes and in particular to the glycosyl hydrolases (GH). The analysis was carried out using the nomenclature of Häkkinen et al. [61]. Of the 9,143 individual genes 228 of these mapped onto carbohydrate active enzymes. The number of genes that returned a RPKM of above 0 was 221, 219 and 198 for days 3, 5 and 7, respectively. Of the 228 identified genes 200 were identified as GH. The number of GH genes returning a RPKM value of greater than 0 was 195, 192 and 175 for days 3, 5 and 7, respectively. It is not possible to analyses all of these genes, however a large number were found to be expressed at very low levels with RPKM values below 10. Also for this study we are really only interested in those genes that are expressed very highly since it is assumed that the high level of RNA will correlate with a high protein expression. It is noted that this need not be the case. In that case an arbitrary threshold for the RPKM of 100 was selected and only those genes with a value in excess of this chosen for analysis. It was 197 found that 28, 38 and 10 of the GH genes fell into this category for day 3, 5 and 7, respectively. The highly expressed GH genes fell into 25 classes according to the CAZy database. The number of genes highly expressed in each class at each of the three time points are shown in Table 3 along with their annotated enzyme activities from the CAZy data base.

Expression pattern for selected CAZy genes

The second analysis was to look at the expression pattern of those CAZy genes thought to be most significant. These were those involved in starch metabolism (GH13-amylase and GH15 glucoamylase), hemicellulose metabolism (GH11-xylanase and GH 74 xyloglucanase) and cellulose metabolism (GH6 cellobiohydrolase and GH7 endo glucanase). The data base was searched for all genes in these 6 categories and the expression levels of these at the three sampling points (Figure 7).

In the case of amylase (GH13) the data base returned four independent genes. These along with the other gene involved in starch metabolism (GH15) showed coordinated expression. In all cases there was relatively high expression at day 3 and this remained relatively constant throughout the sampling period. The two genes involved in hemicellulose expression (GH11 and 74) also showed coordinated expression being low but detectable on day 3 and peaking on day 5. For the genes involved in cellulose metabolism the data base returned two independent genes for endo glucanase (GH7) and the expression of both these was coordinated with the other gene (GH6). In this case expression was extremely low on day 3 and peaked on day 5. The overall pattern is consistent with the hypothesis that the fungus is utilising starch, as the most easily degradable substrate, in the early stages of the fermentation but attempts to exploit the hemicelluloses and then cellulose later on. Expression levels at day 7 were all relatively low suggesting that the fungal population is under severe stress at this stage and maybe dying off.

General highly expressed genes

The top 20 most highly expressed genes were identified for each sampling point. These are listed in Table 4. This list is in ascending order of gene ID number, not in order of expression, to allow easier comparisons between the three time points.

Six genes are represented across all three time points. 2 are common between days 3 and 5 and 11 between days 5 and 7. This supports the global analysis of changes in gene expression where there was a larger variation between days 3 and 5 than between days 5 and 7 and again may reflect the acclimatisation into a steady state.

The JGI WEB site was used to obtain putative enzyme identifications for these genes.

Of the 31 genes identified in the search above 18 returned hypothetical proteins of unknown function. The putative identification of the remaining 13 (Table 5).

As might be expected many of the genes encode for house-keeping genes e.g. ribosomal proteins and histones.

The database can also be searched for expression of specific target enzymes with potential commercial value. Of interest would be lipases and proteases. The *T. reesei* database on the JGI WEB site lists 136 gene IDs as having potential lipase activity and 268 with potential protease activity. A manual search for all of these would not be feasible but future work could explore this further. However, a manual search for the lipase genes has identified at least five that are expressed (Table 6) and one of these has an expression level above 100.

Conclusion

In conclusion, results here show that SSF using MSW as a substrate could represent an economical method for the production of cellulase enzyme with low operational costs as MSW is a cheap and abundant

CAZy group and suggested enzyme activity	Number of genes expressed on day 3	Number of genes expressed on day 5	Number of genes expressed on day 7
GH1 (β-glucosidase)	1	1	0
GH2 (β-mannosidase)	1	1	0
GH3 (β-glucosidase)	0	2	0
GH5 (β-1-3-glucosidase)	1	3	0
GH6 (Cellobiohydrolase)	0	1	0
GH7 (Endo-β-1,4 glucanase)	0	1	0
GH11 (β- 1,4 xylanase)	0	1	0
GH12 (β-1,4 glucanase)	0	1	0
GH13 (amylase)	0	1	0
GH15 (Glucoamylase)	0	1	0
GH16 (glucanosyl transferase)	6	3	1
GH17 (β- 1,3 glucosidase)	3	3	2
GH18 (Chitinase)	1	2	1
GH25 (N.O diacylmuramidase)	1	1	0
GH31 (α- glucosidase)	1	1	0
GH37 (α-trehalase)	0	1	0
GH47 (α-1,2 mannosidase)	1	1	0
GH61 (Cu dependent polysaccharide monooxygenase)	3	3	0
GH71 (α- 1.3 glucanase)	1	1	0
GH72 (β-1,3 glucosyl transferase)	3	3	3
GH74 (Xyloglucanase)	0	1	0
GH76 (α-1,6 mannase)	4	5	2
GH92 (α-1.2 mannase)	1	0	0
GH104 (unassigned)	0	0	1

Table 3: Highly expressed GH genes during *T. reesei* fermentation on MSW (RPKM > 100).

Day 3	Day 5	Day 7
3007	44700	44700
53947	45971	45971
56118	49366	49366
64667	53947	61078
65718	61078	65718
68107	65718	66092
68909	68107	66276
70840	70840	70840
72137	72137	72137
73516	73516	73516
74060	81136	81136
81136	82374	103498
82510	106516	106516
105533	106591	106591
111890	109296	109296
119989	111362	111362
121605	121163	121163
123029	121439	121439
123650	121653	121653
124210	123650	12350

Table 4: Gene ID numbers for the 20 most highly expressed genes at each time point. Green highlights are those identified at all three times. Red those common to days 3 and 5; yellow highlights those common between days 5 and 7.

Gene ID	Putative function
49366	Protein turnover
56118	Acetyl C0 binding protein
68107	Ribosomal protein
68909	Ribosomal protein
73516	Glucose repressible gene protein-related protein
81136	Membrane bound protein of unknown function
82510	Histone H4
106516	Glucose/ribitol dehydrogenase
111890	Phosphate transporter
119989	Hydrophobin 2
121605	Actin regulatory protein
123029	Cu^{2+}/ Zn^{2+} superoxide dismutase
124210	Histone H3

Table 5: Putative functions for the gene products.

Gene ID	EC number	RPKM Day 3	RPKM Day 5	RPKM Day 7
32364	3.1.-.-	11.77651	17.56082	0.417827
66324	3.1.1.23	131.8513	56.24179	22.14281
75989	3.1.-.-	10.71609	13.56596	3.480861
119742	3.1.1.3	36.70678	35.01845	20.04622
56427		10.79305	12.6074	1.494388

Table 6: Expression of selected putative lipase genes in T. reesei during SSF fermentation on MSW.

substrate. *T. reesei* recorded the highest production of cellulase enzyme at 30°C with a 168 hours incubation period using 60% moisture content. Crude enzymes derived from this SSF of MSW were able to release sugars from MSW at a rate similar to that of a commercial enzyme preparation.

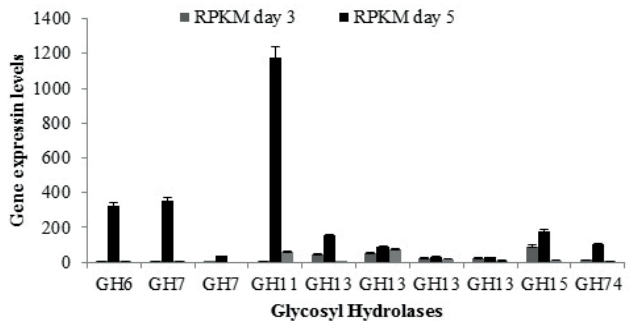

Figure 7: Expression levels for selected GH genes in *T. reesei* during SSF on MSW.

Acknowledgments

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References

1. Vergara SE, Tchobanoglous G (2012) Municipal solid waste and the environment: a global perspective. Annual Review of Environment and Resources. 37: 277-309.
2. Keeling C (2011) Canterbury Region Waste Data Report 2009/2010. Environment Canterbury.
3. Li S, Zhang X, Andresen JM (2012) Production of fermentable sugars from enzymatic hydrolysis of pretreated municipal solid waste after autoclave process. Fuel 92: 84-88.
4. Ray AK, Bairagi A, Ghosh SK, Sen K (2007) Optimization of fermentation conditions for cellulase production by Bacillus subtilis CY5 and Bacillus circulans TP3 isolated from fish gut. Acta Ichthyologica et Piscatoria 37: 47-53.
5. Singh J, Batra N, Sobti RC (2004) Purification and characterisation of alkaline cellulase produced by a novel isolate, Bacillus sphaericus JS1. J Ind Microbiol Biotechnol 31: 51-56.
6. Rajoka MI (2004) Influence of various fermentation variables on exo-glucanase production in Cellulomonas flavigena. Electr J Biotechnol 7: 07-08.
7. Guruchandran V, Sasikumar C (2010) Cellulase production by Aspergillus niger fermented in saw dust and Bagasse. J Cell Tissue Res 10: 2115.
8. Zhao SH, Liang XH, Hua DL, Ma TS, Zhang HB (2013) High-yield cellulase production in solid-state fermentation by Trichoderma reesei SEMCC-3.217 using water hyacinth (Eichhornia crassipes). Afr J Biotechnol 10: 10178-10187.
9. Ncube T, Howard RL, Abotsi EK, Jansen van Rensburg EL, Ncube I (2012) Jatropha curcas seed cake as substrate for production of xylanase and cellulase by Aspergillus niger FGSCA733 in solid-state fermentation. Industr Crops Products 37: 118-123.
10. Singhania RR, Sukumaran RK, Pillai A, Prema P, Szakacs G, et al. (2006) Solid-state fermentation of lignocellulosic substrates for cellulase production by Trichoderma reesei NRRL 11460. Indian J Biotechnol 5: 332-336.
11. Alam MZ, Mamun AA, Qudsieh IY, Muyibi SA, Salleh HM, et al. (2009) Solid state bioconversion of oil palm empty fruit bunches for cellulase enzyme production using a rotary drum bioreactor. Biochem Engineer J 46: 61-64.
12. Latifian M, Hamidi-Esfahani Z, Barzegar M (2007) Evaluation of culture conditions for cellulase production by two Trichoderma reesei mutants under solid-state fermentation conditions. Bioresour Technol 98: 3634-3637.
13. Pensupa N, Jin M, Kokolski M, Archer DB, Du C (2013) A solid state fungal fermentation-based strategy for the hydrolysis of wheat straw. Bioresour Technol 149: 261-267.
14. Couto SR, Sanromán MA (2005) Application of solid-state fermentation to ligninolytic enzyme production. Biochem Engineer J 22: 211-219.
15. Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW (2012) The challenge of enzyme cost in the production of lignocellulosic biofuels. Biotechnol Bioeng 109: 1083-1087.
16. Stutzenberger FJ (1971) Cellulase production by Thermomonospora curvata isolated from municipal solid waste compost. Appl Microbiol 22: 147-152.
17. Gautam SP, Bundela PS, Pandey AK, Khan J, Awasthi MK, et al. (2011) Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. Biotechnol Res Int 2011: 8.
18. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, et al. (2006) Determination of sugars, byproducts, and degradation products in liquid fraction process samples. National Renewable Energy Laboratory.
19. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, et al. (2008) Determination of structural carbohydrates and lignin in biomass. National Renewable Energy Laboratory.
20. Campbell CR (1992) Determination of total nitrogen in plant tissue by combustion. Plant Anal Ref Proc for S US Southern Coop Ser Bull 368: 20-22.
21. Cequier-Sánchez E, Rodríguez C, Ravelo AG, Zárate R (2008) Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. J Agric Food Chem 56: 4297-4303.
22. Hokura A, Matsuura H, Katsuki F, Haraguchi H, et al. (2000) Multielement determination of major-to-ultratrace elements in plant reference materials by ICP-AES/ICP-MS and evaluation of their enrichment factors. Analytical Sciences 16: 1161-1168.
23. Ries L, Pullan ST, Delmas S, Malla S, Blythe MJ, et al. (2013) Genome-wide transcriptional response of Trichoderma reesei to lignocellulose using RNA sequencing and comparison with Aspergillus niger. BMC Genomics 14: 541.
24. Adney B, Baker J (1996) Measurement of cellulase activities. National Renewable Energy Laboratory 1996: 1-6.
25. Barlaz MA, Eleazer WE, Odle WS, Qian X, Wang YS (1997) Biodegradative analysis of municipal solid waste in laboratory-scale landfills. Environmental Protection Agency.
26. Jones KL, Rees JF, Grainger JM (1983) Methane generation and microbial activity in a domestic refuse landfill site. Eur J Appl Microbiol Biotechnol 18: 242-245.
27. Wang YS, Byrd CS, Barlaz MA (1994) Barlaz, Anaerobic biodegradability of cellulose and hemicellulose in excavated refuse samples using a biochemical methane potential assay. J Ind Microbiol 13: 147-153.
28. Barlaz MA, Schaefer DM, Ham RK (1989) Bacterial population development and chemical characteristics of refuse decomposition in a simulated sanitary landfill. Appl Environ Microbiol 55: 55-65.
29. Ham RK, Norman MR, Fritschel PR (1993) Chemical characterization of fresh kills landfill refuse and extracts. J Environ Engineer 119: 1176-1195.
30. Price GA1, Barlaz MA, Hater GR (2003) Nitrogen management in bioreactor landfills. Waste Manag 23: 675-688.
31. Barlaz MA (2006) Forest products decomposition in municipal solid waste landfills. Waste Manag 26: 321-333.
32. Ponsá S, Gea T, Sánchez A (2011) Anaerobic co-digestion of the organic fraction of municipal solid waste with several pure organic co-substrates. Biosystems Engineering 108: 352-360.
33. Rao MS, Singh SP (2004) Bioenergy conversion studies of organic fraction of MSW: kinetic studies and gas yield-organic loading relationships for process optimisation. Bioresour Technol 95: 173-185.
34. Gallert C, Winter J (2005) Bacterial metabolism in wastewater treatment systems.
35. Hartmann H, Ahring BK (2006) Strategies for the anaerobic digestion of the organic fraction of municipal solid waste: an overview. Water Sci Technol 53: 7-22.
36. Roane TM, Rensing C, Pepper IL, Maier RM (2000) Microorganisms and metal pollutants. Environ Microbiol pp: 403-423.
37. Richard TL, Woodbury PB (1992) The impact of separation on heavy metal contaminants in municipal solid waste composts. Biomass and Bioenergy 3: 195-211.

38. Slack RJ, Bonin M, Gronow JR, Van Santen A, Voulvoulis N (2007) Household hazardous waste data for the UK by direct sampling. Environ Sci Technol 41: 2566-2571.

39. Déportes I, Benoit-Guyod JL, Zmirou D (1995) Hazard to man and the environment posed by the use of urban waste compost: a review. Sci Total Environ 172: 197-122.

40. Hasselriis F, Licata A (1996) Analysis of heavy metal emission data from municipal waste combustion. J Hazardous Materials 47: 77-102.

41. Eighmy TT, Eusden JD, Krzanowski JE, Domingo DS, Staempfli D, et al. (1995) Comprehensive approach toward understanding element speciation and leaching behavior in municipal solid waste incineration electrostatic precipitator ash. Environ Sci Technol 29: 629-646.

42. Park YJ, Heo J (2002) Vitrification of fly ash from municipal solid waste incinerator. J Hazard Mater 91: 83-93.

43. Rodella N, Bosio A, Dalipi R, Zacco A, Borgese L, et al. (2014) Waste silica sources as heavy metal stabilizers for municipal solid waste incineration fly ash. Arab J Chem.

44. Wu HY, Ting YP (2006) Metal extraction from municipal solid waste (MSW) incinerator fly ash-Chemical leaching and fungal bioleaching. Enzyme Microbial Technol 38: 839-847.

45. Xu TJ, Ting YP (2004) Optimisation on bioleaching of incinerator fly ash by Aspergillus niger–use of central composite design. Enzyme Microbial Technol 35: 444-454.

46. Mekala NK, Singhania RR, Sukumaran RK, Pandey A (2008) Cellulase production under solid-state fermentation by Trichoderma reesei RUT C30: statistical optimization of process parameters. Appl Biochem Biotechnol 151: 122-131.

47. Mrudula S, Murugammal R (2011) Production of cellulase by Aspergillus niger under submerged and solid state fermentation using coir waste as a substrate. Braz J Microbiol 42: 1119-1127.

48. Maurya DP, Singh D, Pratap D, Maurya JP (2012) Optimization of solid state fermentation conditions for the production of cellulase by Trichoderma reesei. J Environ Biol 33: 5-8.

49. Francis F, Sabu A, Nampoothiri KM, Ramachandran S, Ghosh S, et al. (2003) Use of response surface methodology for optimizing process parameters for the production of a-amylase by Aspergillus oryzae. Biochem Eng J 15: 107-115.

50. Brijwani K, Oberoi HS, Vadlani PV (2010) Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. Proc Biochem 45: 120-128.

51. Bansal N, Tewari R, Soni R, Soni SK (2012) Production of cellulases from Aspergillus niger NS-2 in solid state fermentation on agricultural and kitchen waste residues. Waste Manag 32: 1341-1346.

52. Haq I, Iqbal S, Qadeen M (1993) Production of xylanase and CMC cellulase by mold culture. Pak J Biotechnol 4: 403-409.

53. Nasir Iqbal HM, Asgher M, Ahmed I, Hussain S, et al. (2010) Media optimization for hyper-production of carboxymethyl cellulase using proximally analyzed agroindustrial residue with Trichoderma harzianum under SSF. IJAVMS 4: 47-55.

54. Krishna C (2005) Solid-state fermentation systems-an overview. Crit Rev Biotechnol 25: 1-30.

55. Koppram R, Tomás-Pejó E, Xiros C, Olsson L (2014) Lignocellulosic ethanol production at high-gravity: challenges and perspectives. Trends Biotechnol 32: 46-53.

56. Aiello C, Ferrer A, Ledesma A (1996) Effect of alkaline treatments at various temperatures on cellulase and biomass production using submerged sugarcane bagasse fermentation with Trichoderma reesei QM 9414. Bioresour Technol 57: 13-18.

57. Brijwani K, Vadlani PV (2011) Cellulolytic enzymes production via solid-state fermentation: effect of pretreatment methods on physicochemical characteristics of substrate. Enzyme Res 2011: 10.

58. Kim D, Cho EJ, Kim JW, Lee Y, Chung H, et al. (2014) Production of cellulases by Penicillium sp. in a solid-state fermentation of oil palm empty fruit bunch. African J Biotechnol 13: 145-155.

59. Kannakar M, Ray RR (2010) Extra cellular endoglucanase production by Rhizopus oryzae in solid and liquid state fermentation of agro wastes. Asian J Biotechnol 2: 27-36.

60. Sun H, Ge X, Hao Z, Peng M (2010) Cellulase production by Trichoderma sp. on apple pomace under solid state fermentation. African J Biotechnol 9.

61. Häkkinen M, Arvas M, Oja M, Aro N, Penttilä M, et al. (2012) Re-annotation of the CAZy genes of Trichoderma reesei and transcription in the presence of lignocellulosic substrates. Microb Cell Fact 11: 134.