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Solid substrate fermentation of cassava fibrous residue for production of α-amylase, lactic acid and ethanol

Ramesh C. Ray*, Sabita Mohapatra, Shrutirupa Panda and Shaktimay Kar

Central Tuber Crops Research Institute (Regional Centre), Bhubaneswar - 751 019, India

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Abstract: *There is serious concern about the disposal of solid residues left after large scale extraction of starch from cassava. Owing to the high starch content (55-65% on dry weight basis) and organic matter of these wastes, an attempt has been made to utilize it for the production of three bioproducts, i.e. α-amylase, lactic acid and ethanol in solid substrate fermentation by incubating the solid residue at different moisture holding capacity (40-80%) and incubation period (12- 60 hr for α-amylase, 24- 144 hr for ethanol and 2- 10 days for lactic acid). The highest product yield was obtained at 60% moisture holding capacity of the residue and period of incubation varied from 36 hr (α-amylase), 120 hr (ethanol) to 6 days (lactic acid). This study showed that the solid residues from cassava starch factories could serve as a low-cost substrate for bioproducts production.*

Key words: *α-amylase, Cassava fibrous residue, Ethanol, Lactic acid, Solid substrate fermentation*
PDF of full length paper is available with author ***(*rc.ray6@gmail.com)***

Introduction

In the manufacture of starch from cassava (*Manihot esculenta* Crantz), four types of wastes are produced: outer skins, inner rinds, waste water and fibrous residues (Ray and Ward, 2006). The outer skin, inner rinds and waste water are used for production of biogas, single -cell proteins and yeast single-cell proteins, respectively (Sriroth *et al*., 2000; Adeyemi and Sipe, 2004; Tung *et al*., 2004). The fibrous residue [hereafter referred to as cassava fibrous residue (CFR)] constitutes about 15-20% by weight of the processed cassava chips / tubers, is retained on sieves during the rasping process. It contains about 55-65% starch (on dry weight basis) (Sriroth *et al*., 2000; Jyothi *et al*., 2005); however, because of the difficulty in the disposal of solid residues it leads to serious environmental problems (Ray, 2004).

Owing to the high starch content and organic nature (rich in nutrient) of these wastes, CFR can serve as an important substrate for production of various bioproducts, *i.e.* amylase enzyme (Ray, 2004), glutamic acid (Jyothi *et al*., 2005), xanthan gum (Woiciechowski *et al*., 2004) *etc.* In this paper, preliminary studies (effect of incubation period and effect of moisture holding capacity) have been carried out for the production of (1) thermostable α-amylase, (2) lactic acid and (3) bioethanol by solid substrate fermentation (SSF) of CFR.

Materials and Methods

Microorganisms: The microorganisms used in this study were *Bacillus brevis* MTCC 7521, *Lactobacillus plantarum* MTCC 1407 and *Saccharomyces cerevisiae* RC CTCRI for α-amylase, lactic acid and ethanol production, respectively. *Bacillus brevis* MTCC 7521 strain was earlier isolated from the brick kiln soil nearby Bhubaneswar, India and subsequently identified at the Institute of Microbial Technology, Chandigarh, India and given the Code No. MTCC 7521. The other two microorganisms *i.e., Lb. plantarum* MTCC 1407 and *S. cerevisiae* RC CTCRI were obtained from Institute of Microbial Technology, Chandigarh and Microbial Culture Collection of Regional Centre, Central Tuber Crops Research Institute (CTCRI), Bhubaneswar, India, respectively. The strains *B. brevis* and *Lb. plantarum* were maintained in starch-beef extract (SB) and mann-rogassa-sharpe (MRS) medium respectively at 4°C. The yeast *S. cerevisiae* was maintained on potato dextrose agar medium at 4°C.

Cassava fibrous residue (CFR): CFR was used as solid substrate (support and nutrient source) for SSF. CFR was collected during starch extraction from cassava using the mobile starch extraction plant, developed by our institute (Balagopalan, 2000). Because of its high water content (70- 80%) and presence of high quantity of starch (63% on dry weight basis), the residues were de-watered, sun-dried for 6-8 days and then oven-dried at 80°C for 24 hr to prevent microbial deterioration. The dried CFR was stored in air-tight container at room temperature (30 ± 2°C) until required. The chemical constituents of CFR were given in Table 1(Ray, 2004).

Amylase production: The inoculum was prepared in SB broth (soluble starch, 2.0%; beef extract, 1%; $MgSO_4$ 0.01%; $CaCl_2$, 0.02%; pH, 7.0) by transferring a loop full of the organism (*B. brevis*) from a stock culture and incubating at 50°C and 120 rpm for 24 hr in an orbital incubator shaker (Remi India Pvt. Ltd., Bombay, India).

Roux bottles (132 mm x 275 mm) containing 20 g of CFR were moistened with 27 ml of distilled water [to provide 60% moisture holding capacity (MHC)] containing 10% beef extract (as nitrogen source) were autoclaved at 15 lb pressure for 30 min, then cooled and inoculated with 10% (v/w) inoculum [2 ml (1 x 10^7 CFU /ml)]. The bottles were incubated horizontally at 50°C in an incubator for

60 hr. The contents in the Roux bottles were periodically mixed by gentle tapping. At 12 hr interval the bottles in duplicate were taken out and the enzyme content was extracted with 35 ml of distilled water added to the solid substrate (CFR) and squeezed through a wet cheese cloth. The pooled enzyme extract was centrifuged at 8000 rpm for 20 min and the clear supernatant (volume adjusted to 25 ml with distilled water) was used for the enzyme assay.

The effect of moisture levels on the enzyme titre were evaluated by varying the moisture content of CFR from 40 to 80% of MHC, adjusted by altering the amount of medium used to moisten the substrate and the samples were incubated at 50°C for 36 hr.

Table - 1: Biochemical constituents of cassava fibrous residue (Ray, 2004)

Constituents	CFR (g/100 g dry residues)
Moisture	11.2
Starch	63.0
Crude fibre	10.8
Crude protein	0.88
Free reducing sugars	1.45
Hydrocyanic acid	0.008
Total ash	1.2

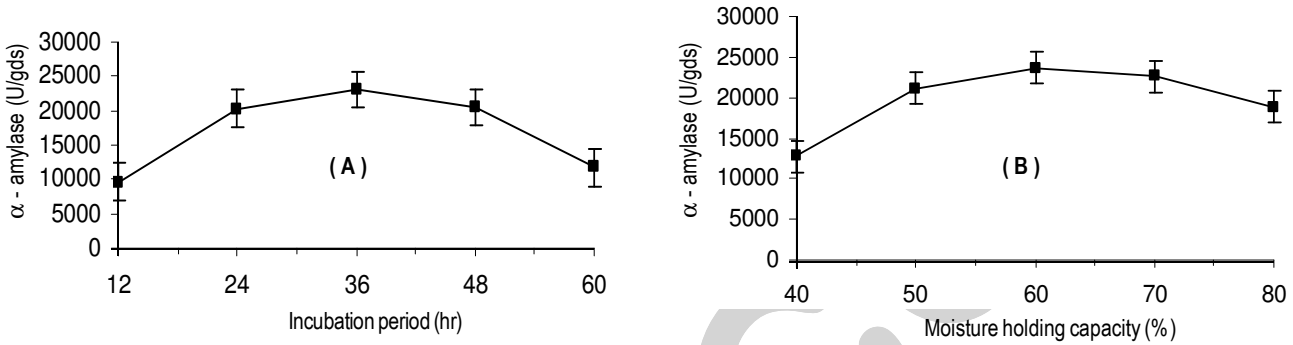

Fig. 1: α-amylase production by *Bacillus brevis* MTCC 7521 by SSF of cassava fibrous residue
A = Effect of incubation duration, B = Effect of moisture holding capacity, ⊥■⊥ = Standard error

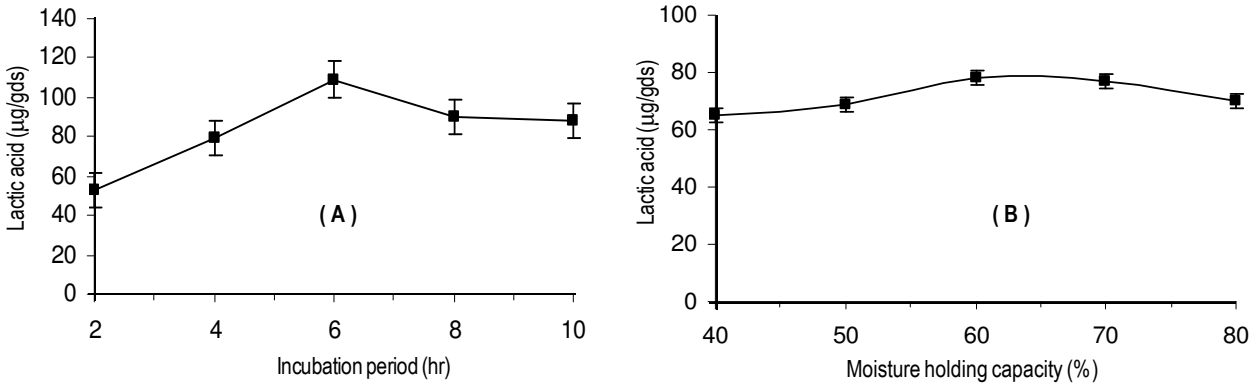

Fig. 2: Lactic acid production by *Lactobacillus plantarum* MTCC 1407 by SSF of cassava fibrous residue
A = Effect of incubation period, B = Effect of moisture holding capacity, ⊥■⊥ = Standard error

The amylase assay was based on the reduction of blue colour intensity resulting from enzymatic hydrolysis of starch and formation of starch–iodine complex (Palanivelu, 2001). The reaction mixture consisted of 0.2 ml of microbial enzyme, 0.25 ml of 0.2% starch solution and 0.05 ml of phosphate buffer incubated at 50°C for 10 min. The reaction was stopped by adding 0.25 ml of 1N HCl and the colour was developed by adding 0.25 ml of I / KI solution (2% KI in 0.2% I). The O.D (optical density) of the blue colour solution was determined using a UV- Vis spectrophotometer at 690 nm. One unit of enzyme activity is defined as the quantity of enzyme that causes 0.01% reduction of blue colour intensity of starch iodine solution at 50 °C per minute per ml (Palanivelu, 2001). The enzyme unit was expressed as units / gram dry substrate (gds).

Lactic acid (LA) production: The inoculum was prepared in MRS broth (g/l: peptone, 10.0; beef extract, 10.0; yeast extract, 5.0; glucose, 20.0; Na_2HPO_4, 2.0; sodium acetate, 5.0; tri-ammonium citrate, 2.0; $MgSO_4$, 0.2; $MnSO_4$, 0.2; pH 6.2-6.6) by transferring a loop full of organism (*Lb. plantarum*) from a stock culture and

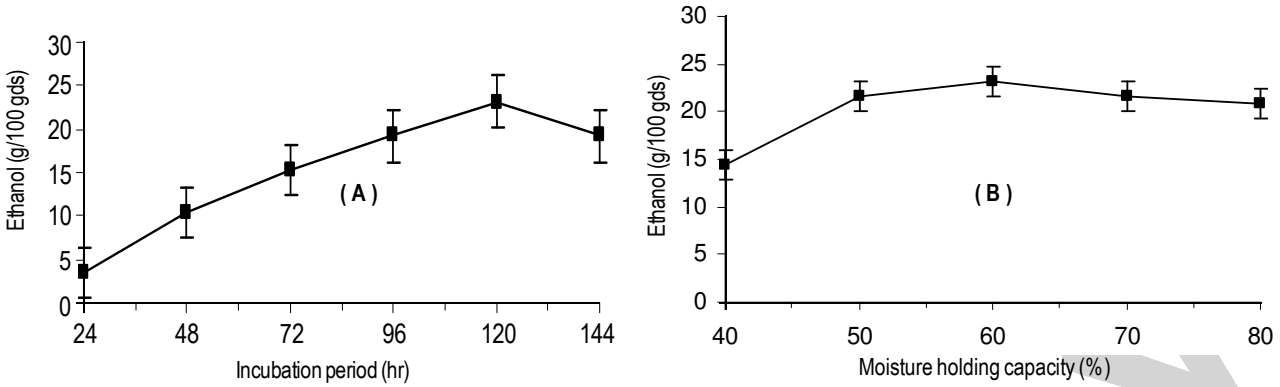

Fig. 3: Ethanol production by *Saccharomyces cerevisiae* RC CTCRI by SSF of cassava fibrous residue.
A = Effect of incubation period, B = Effect of moisture holding capacity, ⊥■⊥ = Standard error

incubating at 30°C and 120 rpm for 48 hr in an orbital incubator shaker (Remi India Pvt. Ltd. , Bombay, India).

Roux bottles (132mm x 275 mm) containing 20 g of CFR were moistened with 27 ml of distilled water (to provide 60% MHC) containing 10% beef extract (as nitrogen source) were autoclaved at 15lb pressure for 30 min, then cooled and inoculated with 10% (v/w) inoculum [2 ml (1 x 10^6 CFU/ml)]. The bottles were incubated at 35°C for 10 days. At two days interval, the bottles in duplicates were taken out and LA content was extracted by adding 35 ml distilled water to CFR. Then the water mixed substrate was squeezed through a wet cheese cloth. After extraction, the pooled LA source was centrifuged at 8000 rpm for 20 min and the supernatant (volume adjusted to 25 ml) was taken for LA estimation.

The effect of initial moisture content on LA production was studied by varying the moisture content of CFR to 40-80% MHC, adjusted by altering the amount of medium used to moisten the substrate and the samples were incubated for 4 days at room temperature, 30 ± 2°C.

LA content was estimated by the method described by Amerine and Ough (1984) using a UV-Vis spectrophotometer and expressed as µg LA/gds.

Ethanol production: The yeast (*S. cerevisiae*) was grown first in 250-ml Erlenmeyer flasks containing 100 ml sterilized medium (yeast extract- nutrient broth) with sugar concentration 12% (w/v) and the pH was adjusted to 5.5 by dilute 1 N HCl. It was cultured for 24 hr at 30°C in an incubator. This served as the starter culture for ethanol production.

Fifty g of CFR was taken in Roux bottle (132 mm x 275 mm), moistened with 68 ml of water to effect 60% MHC. Then 0.05 ml (0.1%) of Termamyl^R (Novozyme, Denmark) was added to the moistened CFR, mixed thoroughly and incubated at 90°C for 1hr. Then the Roux bottles were taken out and cooled at room temperature, 30 ± 2°C. After partial dextrinization of CFR using Termamyl^R, the enzyme amyloglucosidase (AMG^R Novozymes, Denmark) at 2% level was added to the individual bottles, thoroughly mixed and incubated for 24 hr at 45°C in a BOD incubator for saccharification of starch/dextrin into fermentable sugars. Then the yeast starter culture [10% v/w (2x 10^8/ CFU/ml)] was added to each bottles containing saccharified CFR and kept for fermentation at room temperature (30 ± 2°C) with periodical mixing of substrate.

The effect of moisture level on ethanol production was studied by varying the MHC of CFR from 40 to 80% and incubated for 120 hr at room temperature 30 ± 2°C.

At interval of 24 hr, the fermented substrate was mixed with tap water (1:5 w/v) and the whole mash was distilled to collect the ethanol. The ethanol concentration of the fermentation liquid was determined by measuring the specific gravity of the distillate by the procedure described by Amerine and Ough (1984). The ethanol yield was expressed as g ethanol/100 gds.

Results and Discussion

The nutrient and starch profile of CFR from cassava starch industries vary because of the lack of standard procedure. This variability in nutrient and starch quantity is attributed to several factors such as cassava tuber washing process, the ratio of tuber to water used in the extraction process and the quality of raspers used for starch extraction (Ray and Ward, 2006). In general, a starch content of 55-65% (dry weight basis) is found in CFR surveyed from several factories in India (Jyothi *et al*., 2005; Ray and Ward, 2006).

α-amylase production: *Bacillus brevis* MTCC 7521 produced amylase optimally at its optimum growth temperature of 50°C . Using thin- layer chromatographic analysis, it was ascertained that the amylase produced by the bacterial culture was α-amylase owing to the presence of maltose and glucose as the main end products of starch hydrolysis. Further, the maximum amylase production (23050 units/ gds) was obtained at 60% MHC and at 36 hr of incubation (Fig. 1A, B). When the moisture level increased beyond 60% level, the enzyme activity started decreasing. This decline might be attributed to poor aeration in solid state and adsorption of enzyme to the substrate particle. Similar findings have been reported by other researchers (Pandey *et al*., 2000a; Ray *et al*., 2006).

It is generally regarded that moisture levels of 55-60% are suitable for microbial enzyme production in SSF, when agro-industrial wastes like cassava and sugarcane bagasse, rice bran, wheat floor, fruit pomaces, *etc.* are used as substrates (Pandey *et al*., 2000 a,b,c). Likewise, optimum incubation period for most enzymes vary from 36-96 hr, depending on the environmental conditions (Pandey *et al.,* 2000a). For *Bacillus* sp, optimum range of incubation period for various enzyme production was as follows: amylase (36-60 hr) Babu and Satyanarayana (1995), Malhotra *et al*. (2000), pectinases (48-60 hr) Kapoor and Kuhad (2002), Kashyap *et al*. (2003), lipases (24-48 hr) Abdel-Fattah (2002), Kumar *et al*. (2005) *etc*.

Lactic acid (LA): LA is mostly used for the manufacture of emulsifiers and as additives in food industry. LA can be produced chemically or by microorganisms (lactic acid bacteria) (Pai, 2003). However, most lactobacilli cannot use starch for bioconversion into LA. *Lactobacillus amylophilus*, *Lb. amylovorans* (Zhang and Cheryan, 1991; Naveena *et al*., 2004) and few strains of *Lb. plantarum* (Giraud *et al*., 1991, 1993) are able to ferment starch into sugar and consequently to LA. The strain *Lb. plantarum* MTCC 1407 is amylolytic and has been used in our laboratory as starter culture for the preparation of curd and pickles from sweet potato (*Ipomoea batatas* L.) (Panda *et al*., 2006) in which formation of LA is the principal organic acid responsible for sourness in the fermented foods. In the present study, CFR at 60-70% MHC produced maximum LA (77.89 µg /gds) (Fig. 2 B); the results being concomitant with other studies on LA production using agricultural waste residues as substrates, *i.e.* cassava (Pandey *et al*., 2000c), sugarcane bagasse (Pandey *et al*., 2000b), alfalfa and soya fibre, corn cob and wheat straw (Sreenath *et al*., 2001), *etc*. Further, LA production increased linearly with the days of incubation, up to six days; thereafter, a gradual decline was observed (Fig. 2A).

Ethanol: The ethanol production from CFR is shown in Fig. 3. The peak ethanol production was obtained at 120 hr of fermentation which declined when incubation period further increased to 144 hr. The initial starch content was about 600 g/ kg CFR (equivalent to 666 g/ kg of fermentable sugar assuming 100% saccharification by treatment of Termamyl^{R} and AMG^{R}). However in reality maximum 85 -95% saccharification of cassava starch to sugar by application of Termamyl^{R} and AMG^{R} have been reported (Woiciechowski *et al*., 2002). The peak ethanol yield of 232 g /kg CFR shows that nearly 80% conversion of fermentable sugar into ethanol has been achieved assuming 90% conversion of starch in CFR to fermentable sugars [45 kg of fermentable sugar (as glucose) yield 23-25 kg of ethanol] (Reed , 2002). Further, highest ethanol yield was observed at 60% MHC of CFR.

SSF may be advantageous in production of ethanol from agricultural residues such as CFR because it can reduce fermentor volume and distillation costs (Pandey *et al.*, 2000a; Ward and Singh, 2002). Examples of residues which have been used in this manner include apple pomace (Nagadi and Correia, 1992), sorghum (Henk and Linden, 1996) and carob pods (Roukas, 1994). In these studies, maximum ethanol production was obtained at 60-70% moisture holding capacity of the substrate. However, previous studies using submerged fermentation have found ethanol production from CFR was not economical because the fermentable sugar in starch hydrolysate never exceeded 8-10% (w/v), which requires fortification of molasses to raise sugar concentration to 15% (w/v) (Kunhi *et al*., 1981). However, such problems are not encountered in SSF as evidenced from our study.

In India, more than 1500 cottage and small industries crush over 5000 tonnes of cassava per day during harvest season (Jyothi *et al*., 2005) for the manufacture of starch and sugar, thereby generating 1000 tonnes of dry residue equivalent per day. The residue, if effectively used, could lead to any one of the arrays of value added products such as enzyme (α-amylase), LA and ethanol. There are reports for production of some value-added products *i.e.* amylases (Ray, 2004), fructose syrup (Srikanta *et al*., 1989), glutamic acid (Jyothi *et al*., 2005), xanthan gum (Woiciechowski *et al*., 2004), *etc*. by solid/ submerged fermentation of CFR. Enzymes (α-amylase), lactic acid and ethanol are some other examples of bioproducts from CFR by microbial fermentation. Utilization of CFR at the place where it is generated will also eliminate any expenditure on transport.

There are several important factors which affect SSF processes. Among these, selection of suitable strain, substrate and process parameters (physical chemical and biochemical) are crucial (Pandey *et al*., 2000a). Although the present studies are preliminary, despite that the microbial strains and substrate (CFR) used in this study were found suitable for production of these bioproducts (α-amylase, LA and ethanol). Further research is in progress in our laboratory to optimize the various process parameters for increasing yield of these products in SSF.

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