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Design of Experimental Lab Scale Vertical Mass Flow Type Bioreactor for Bioethanol Production by Co-Culture Strategy from Cassava Waste

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Abstract

Due to a surge in demand and cost, there is a requirement for alternative energy sources by devising strategies for the efficient production of biofuels. Immobilized microbial systems for the conversion of biomass to fuels have become progressively important. Cassava (*Manihot esculentum*) processing waste, a massive byproduct of starch processing is utilized in this work for bioethanol production. This study was an attempt to design and develop an experimental lab-scale Vertical Mass-Flow type Bioreactor (VMFB) demarcated into aerobic and anaerobic zones to produce bioethanol. The upper aerobic zone was meant for saccharification and the lower anaerobic zone for fermentation, the technique is called Simultaneous Saccharification and Fermentation (SSF). The feasibility of co-immobilizing saccharification strains (*A. awamori and D. bruxellensis*) and fermentation strains (*Z. mobilis*) for bioethanol production through SSF from cassava agro-waste were tested. Polyurethane foam was used in the aerobic reaction zone and calcium-alginate beads immobilized microorganisms in the anaerobic reaction zone were employed as carriers for the immobilization. The main objective of this study was to understand the usability of agricultural waste, especially cassava processing waste as raw material for bioethanol production, using SSF technology a concentration of 8 % w/w ethanol was obtained.

Keywords: Bioethanol; Cassava processing waste; Co-immobilization; SSF; VMFB.

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1. Introduction

Conventional energy resources could hardly meet the increasing energy demands; hence, biofuels such as bioethanol have turned into promising alternatives to fossil fuels. Bioethanol is being exploited as one of the prominent bioenergy involving many government and self-funded organizations interested as they have many advantages such as high-octane fuel, and it reduces polluting emissions as well. In this work, starch was chosen as a biomass resource, because of its local availability and rich content in a wide

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range of plants including cassava, wheat, potato, and rice [1]. Cassava is an industrial crop that is processed for several products and yields half a ton of fibrous residue as waste during several processing steps [2]. The major components of cassava waste are starch (50 % dry weight), cellulose, hemicellulose, and ashes. Cassava waste, an agricultural byproduct can offer huge opportunities for ethanol production due to the enormous availability and inexpensive raw material in cassava-growing countries [3].

Several operational processes have been successfully used in practice for commercial bioethanol production, such as fed-batch, repeated batch; continuous process; Melle-Boinot process; and membrane systems. Many bioreactor configurations air-lift bioreactors, single- or two-stage packed column bioreactors, and column bioreactors coupled with or without settlers, are obtaining promising results [4]. The main aspect of our work is to showcase one such configuration methodology employed in the designing, fabricating, and experimental set-up of a vertical mass flow bioreactor consisting of both aerobic and anaerobic zones and thus developing technology for Simultaneous Saccharification and Fermentation (SSF). The economical lab-scale prototype developed was also analyzed for bioethanol production. Such a type of reactor can be easily controlled for optimizing the fermentation environmental parameters and conditions for high yields of biomass.

From various experimental reports, it was found that *Zymomonas mobilis* produce ethanol at more than double the concentration than reported rates for yeasts, with developed process systems that have demonstrated the potential of this microorganism for industrial bioethanol production [5]. The experimental investigations were conducted on ethanol production with immobilized whole cells**.** The work was also to investigate the feasibility of co-immobilizing saccharification strains (*Aspergillus awamori and Dekkera bruxellensis*) and fermentation strains (*Z. mobilis*) for bioethanol production through SSF of cassava starch. Polyurethane foam (aerobic reaction zone) and calcium-alginate beads (anaerobic reaction zone) were employed as carriers for the immobilization [6]. Our work is a combinatorial approach for the low-cost, green strategy for bioethanol production encompassing several spheres such as the use of the fabricated prototype of bioreactor; rapid substrate conversion; use of multiple microbial strains, and immobilized fermentation microbial strains.

2. Materials and Methods

2.1. *Microorganisms and media*

Three microbial cultures namely *Aspergillus awamori* (MTCC 8002), *Dekkera bruxellensis* (MTCC 540), and Zymomonas *mobilis* (MTCC 88) were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh-160036 (India). The cells were revived using appropriate media.

2.2. *Collection and pretreatment of cassava waste*

Cassava waste was collected, and sun-dried for 7 days to remove moisture. The dried cassava peel was powdered with a blender and sieved through a 2 mm sieve in a sieve shaker and added to the fermentation media (Fig. 1).

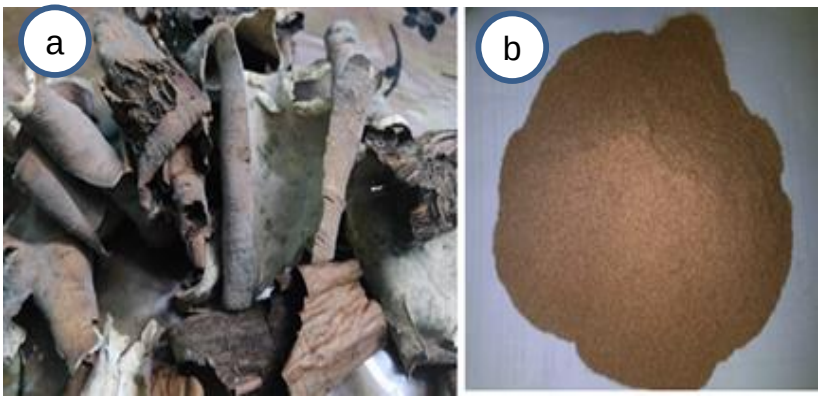

Fig. 1. Digital images of dried (a) Cassava peels and (b) peel powder.

2.3. *Culturing of microorganisms*

The suspension cultures of both *A. awamori* and *D. bruxellensis* were aseptically grown on potato dextrose agar (PDA); incubated at 30 °C for 5 days until sporulation and stored at 4 °C (Fig. 2). The suspension culture of *Z. mobilis* was cultured in the plate consisting of yeast extract (5.0 g/L), reducing sugar (20.0 g/L) and agar (20 g/L), incubated at 30 ˚C for 2 days. The precultural media consists of yeast extract (10.0 g/L), KH_2PO_4 (1.0 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $(NH_4)_2SO_4$ (1.0 g/L), glucose (100.0 g/L), potato starch (5.0 g/L) and distilled water. The fermentation media consisted of yeast extract (2.0 g/L), poly peptone (5.0 g/L), KH_2PO_4 (1.0 g/L), $MgSO_4{\cdot}7H_2O$ (1.0 g/L), $FeSO_4.7H_2O$ (0.01 g/L), $CaCl_2.2H_2O$ (2.0 g/L), cassava waste powder (60.0 g/L), and distilled water.

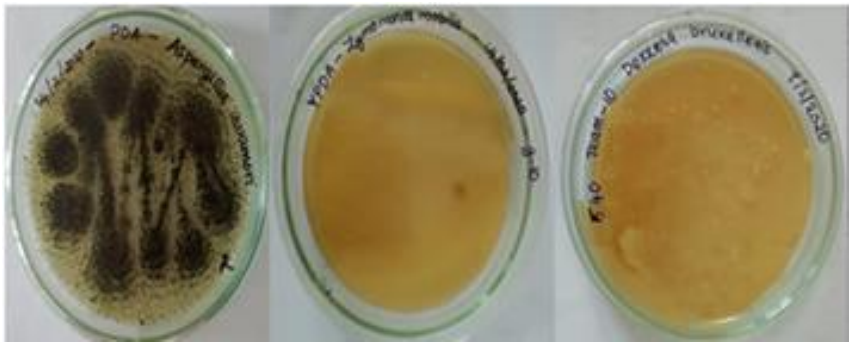

Fig. 2. Standard strains of *Aspergillus awamori*; *Zymomonas mobilis* and *Dekkera bruxellenis.*

2.4. *Designing of bioreactor*

A modified vertical mass flow type bioreactor, VMFB, with an upper aerobic zone and a lower anaerobic zone was designed to enhance bioethanol production (Fig. 3a,b). It has a total working volume of 1.5 L, and the fermentation media were circulated between the aerobic upper and anaerobic lower zone by gravity flow (upper to lower) and pump flow (lower to upper) in the VMFB. The preliminary design and graphical design for VMFB were done using Siemens NX version 10 (Fig. 4).

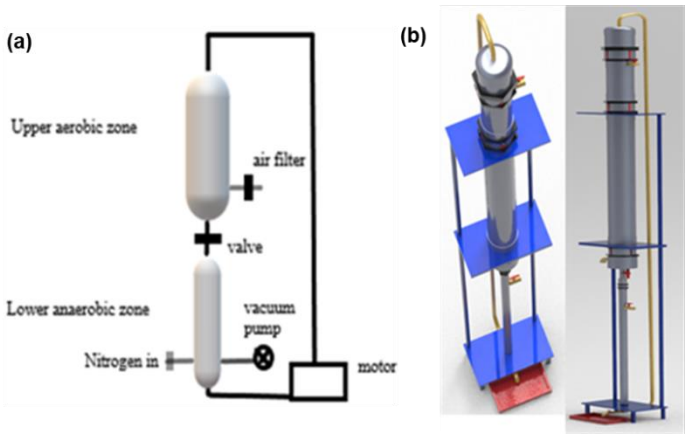

Fig. 3. (a) Schematic diagram of VMFB with divided aerobic and anaerobic reaction zones in the modified bioreactor (Microsoft Paint 3D Windows 10). (b) Preliminary design of VMFB (Siemens NX Version 10 Software).

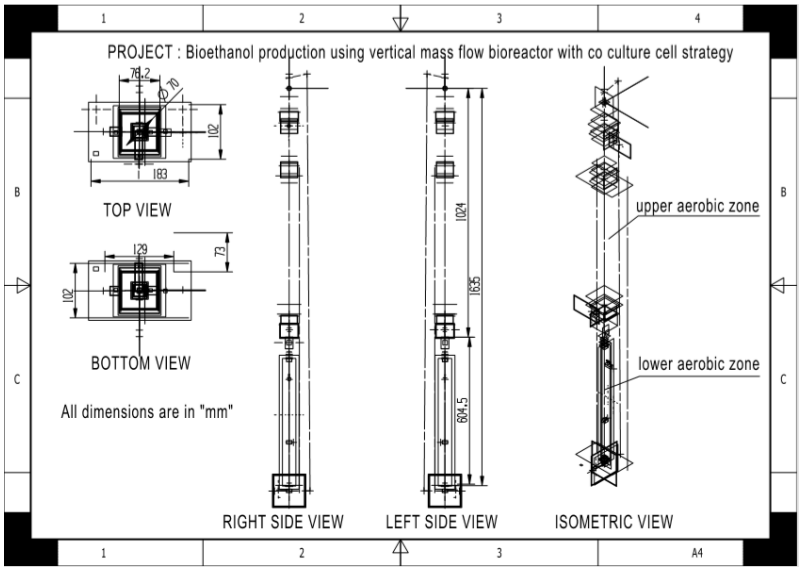

Fig. 4. Graphical design of VMFB (Siemens NX Version 10 Software).

2.5. *Fabrication of bioreactor*

Materials for fabrication of experimental set up of a prototype bioreactor comprising of 2½ (63 mm) PVC 4 Kg pipe – 1M; 63 mm FTA – 3 No; 63 mm MTA – 3 No; 63 mm end cap – 2 No; Press nipple L -4 No; ¾ " PVC 4 K pipe; ¾ " MTA – 4 No; ¾ " FTA- 4No; ¾ " end cap- 3 No; ¾ " PVC bush – 2 No; Teflon tape – 2 No; PVC Compound – 60 ml cap – 2 No; Press nipple L- 4 No; ¾ " PVC 4 Kg pipe; ¾ " MTA – 4 No; ¾ " FTA- 4 No; ¾ " end cap- 3 No; ¾ " PVC bush – 2 No; Teflon tape – 2 No; PVC Compound – 60 mL.

2.6. *Cell immobilization and cultivation*

The *Z. mobilis* cells were mixed with sodium alginate to yield a 4.0 % (w/v) Na-alginate solution. The alginate beads are prepared by thoroughly mixing the 3 % sodium alginate solution and injecting it into a 3 % $CaCl_2$ solution with a needle. Finally, the alginate beads containing bacteria are harvested by washing them with a phosphate buffer solution. After gelling, the microbeads are placed in double distilled water to remove unreacted material. Micro-beads with cells are stored in a physiological solution at 8 °C. Concentrated aqueous *D. bruxellensis* and *A. awamori* spores were used to inoculate in 500 mL Erlenmeyer flasks containing 200 mL of precultural media and agitated at 200 rpm orbital shaker at 30 °C (Fig. 5a). The *D. bruxellensis* and *A. awamori* spores were mixed and then cultivated in a polyurethane (PU) carrier (Fig. 5b) in the upper aerobic reactor of a co-immobilized system.

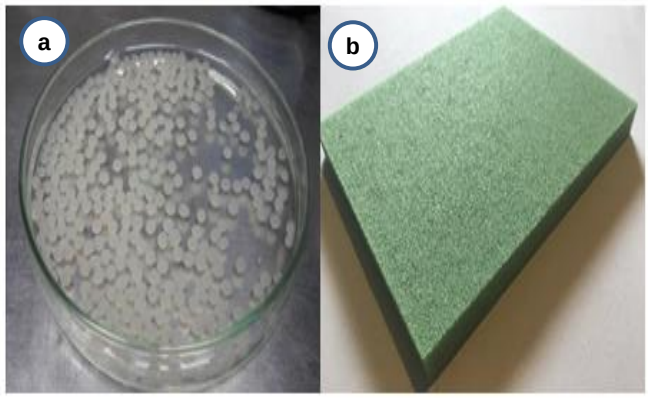

Fig. 5. (a) Immobilized cells in alginate beads and (b) polyurethane foam as a carrier.

2.7. *Ethanol production using VMFB*

The bioreactor comprises one pump without an air compressor to allow energy saving in a simple structure, and an easy operation for ethanol production by simultaneous saccharification and fermentation of cassava waste using a co-cultural microbial system. Reducing sugar from the upper aerobic reactor was used as the substrate for bioethanol

production in the lower anaerobic zone. Briefly, bioethanol was produced in a novel bioreactor by co-cultivating three strains, *D. bruxellensis*, *A. awamori*, and *Z. mobilis*. First, in the pre-cultivation step, both *D. bruxellensis* and *A. awamori* were co-cultured in the upper aerobic zone with fresh precultural media full of fungi on a polyurethane carrier for 3 days. The PU carrier served as a fungal carrier and an enzyme filter during bioethanol production. Meanwhile, *Z. mobilis* is entrapped into the alginate beads. After pre-cultivation, the second step was to remove the pre-cultural media and then put the microbial-laden alginate beads in the lower anaerobic zone. Fresh fermentation medium for bioethanol production was then fed into the modified bioreactor, and the substrate saccharification and ethanol fermentation were simultaneously performed.

2.8. *Lab scale studies*

At first, *D. bruxellensis* and *A. awamori* were cultured in a 500 mL Erlenmeyer flask containing sterilized fermentation media for 48 h at 30 °C. After 48 h of culturing, *Z. mobilis* were added to the flask under aseptic conditions and incubated for 3 days to obtain a better yield. Along with this, two other flasks, one containing *D. bruxellensis* and *A. awamori*, and the other one containing *Z. mobilis* were tested for better yield. After 3 days of incubation, the products including microorganisms were centrifuged at 1000 rpm for 10 minutes. The supernatant was subjected to distillation. The distillation unit was operated at 80 °C for a duration of 30 min. The distillate was collected and measured (Fig. 6).

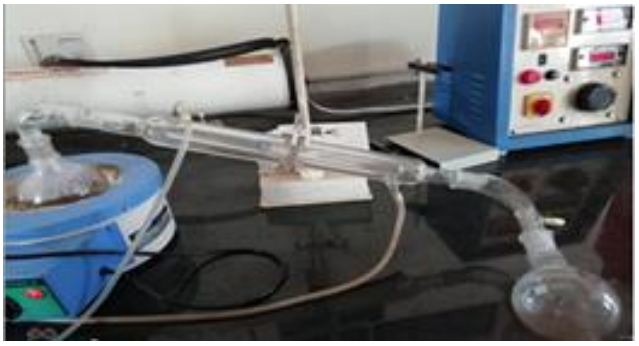

Fig. 6. Experimental set-up of distillation to obtain ethanol.

2.9. *Estimation of reducing sugar*

The estimation of alcohol was done indirectly by measuring the decrease in reducing sugar content. During the fermentation, the broth was estimated for reducing sugar content by the DNS method refers to the theory of Miller (1959) [7]. The concentration of reducing sugar/ml is determined using the standard graph. The Iodoform test confirmed the presence of ethanol and ethanol concentration was determined refractometrically [8].

3. Results and Discussion

3.1. *Designing and fabrication of prototype of VMF bioreactor*

The main body of VMFB was fabricated from PVC pipes and machined in accordance with designed features (Fig. 7). The two zones were bonded using clamping PVC plates and Teflon tapes were used to seal the surface. Tubing's are provided for the addition of fermentation media and the other end has a provision to attach to a pump for media circulation.

Fig. 7. Fabricated VMFB with the lower anaerobic and upper aerobic zone.

3.2. *Efficiency of co-cultivated A. Awamori and D. Bruxellenis in suspension cultivation*

To evaluate the co-culturing of microorganisms in the efficient conversion of starch to reducing sugars, the effects of co- and single microorganism systems were studied. To enhance the saccharification enzyme activity, *A. awamori* and *D. bruxellenis* were co-cultivated. The results implied the synergistic effects of *A. awamori* and *D. bruxellenis* for the accumulation of reducing sugars (Table 1). Fermentation media was used as blank and the concentration of reducing sugar/mL is determined using the standard graph of glucose of the DNS method (Fig. 8).

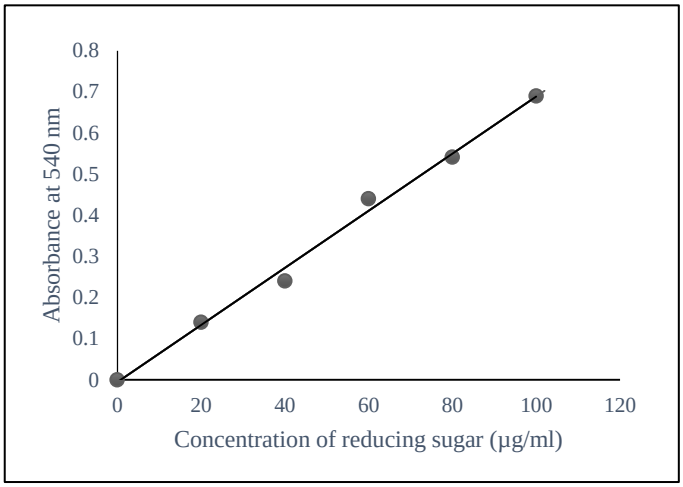

Fig. 8. Standard graph for the determination of reducing sugar by DNS method

Table 1. Effect of single and co-microorganisms on starch hydrolysis.

Sample No.	Microorganisms	Concentration of reducing sugars (µg/mL)
T1	Pure culture of *A. awamori*	64.21
T2	Pure culture of *D. Bruxellenis*	69.86
T3	*Co-culture of A. awamori* and *D. bruxellenis*	103.78

3.3. *Effect of co-suspended and co-immobilized A–D system on the SHF of ethanol from cassava starch*

To investigate the effectivity of immobilization culture on the saccharification step by *A. awamori* and *D. bruxellenis*, co-suspension and co-immobilization cultures were performed and compared on SHF of ethanol from cassava starch. After saccharification culture, co-suspension of *A. awamori* and *D. bruxellenis* (A-D) cultured for 18 h at 200 rpm, the gel immobilized *Z. mobilis* was added into the system, and SHF was performed at 120 rpm in the co-suspended A-D system. On the other hand, saccharification (co-immobilized A-D, 180 rpm) and fermentation (gel-immobilized *Z. mobilis*) were connected and circulated. The maximum ethanol production and productivity in the co-suspended and co-immobilized A-D system were 2.99 g/L, 0.06 g/L h, and 17.7 g/L, 0.21 g/L h, respectively. The results indicated that the co-immobilized cultivation system had better effects and stability than the co-suspension system for co-cultured A-D, and consequently increased the ethanol yield as well as time efficiency.

3.4. *Batch ethanol production on the SSF from cassava waste peel using a single-stage system by VMFB*

A single-stage simultaneous co-fermentation process using VMFB was performed and compared with the traditional two-stage hydrolysis and fermentation. After *D. bruxellenis* and *A. awamori* were co-immobilized and precultured using a PU carrier in the upper aerobic zone for 3 days, the 24 h precultured gel-immobilized Z. *mobilis was* poured into the lower anaerobic zone and bioethanol production was performed with fresh fermentation media. The results of the experiments in batch processes clearly emphasized that the single-stage SSF system using VMFB is advantageous in comparison with the traditional two-stage SHF system using shake flasks for its simple operation. In the iodoform test, cloudy yellow precipitate formation confirmed the presence of ethanol (Fig. 8).

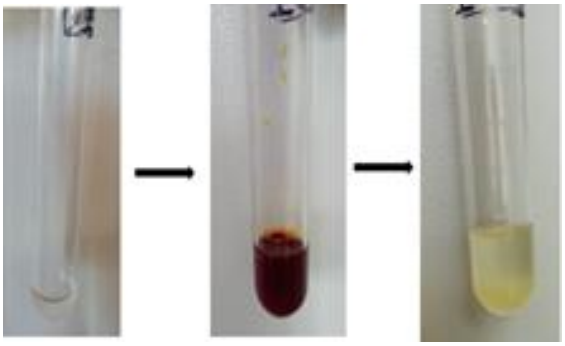

Fig. 8. Iodoform test showing yellow precipitate.

3.5. *Concentration of ethanol*

The concentration of ethanol was determined refractometrically. The value was compared with the standard table containing the concentration of ethanol and the corresponding refractive index. The refractive index obtained is 1.3381 so the concentration of ethanol was 8 % w/w.

Agro-based byproducts as being used as an alternate substrate for the commercial production of several industrially important products [9]. Based on the type of feedstock used, industrial bioethanol production is divided into three generations and approximate ethanol yields from different types of feedstocks have been studied extensively [10]. Bioethanol production by utilizing cassava peels waste through enzymatic and microbiological hydrolysis is up to 3.76 % [11]. Comparative studies are carried out between different types of fermentor configurations yielding higher products. Immobilized cell technology exhibits better mass transfer at high cell densities and better capability to run continuous fermentation with shortened reaction time compared with suspension cell technology [12]. An innovative fermentation scheme was designed, co-culturing immobilized *Zymomonas mobilis* and free cells of *Pichia stipitis* in a modified

fermentor for glucose and xylose fermentation, respectively [13]. Similar work was conducted by Liu *et al*. on bioethanol production from potato starch by a novel vertical mass-flow type bioreactor with a co-cultured-cell strategy, with ethanol production of 60.18 g/L [14]. Waste from cotton textiles was converted to bioethanol via simultaneous saccharification and fermentation (SSF) [15]. Bioethanol produced from cellulase-producing bacteria from soils of the agro waste field was qualitatively proved by iodoform assay [16].

4. Conclusion

A prototype of a bioreactor was developed by using a special space design to connect the two separated aerobic and anaerobic segments. The SSF process of aerobic saccharification and anaerobic fermentation has the advantage of reducing the cost and simplified apparatus. In the lower anaerobic zone, stirring was only by gravity flow circulation instead of any pump. The two stages of the traditional design of ethanol production from agro wastes such as saccharification and fermentation can be overcome by vertical gravity flow from the bioreactor, VMFB. The bioreactor designed was a matrix for bioethanol production, with different co-culture models and policies. This work aimed at producing ethanol by cost effective method to meet the increasing demand for biofuels. The future perspective would be fabricating designed VMFB in stainless steel and optimizing the production medium constituents and conditions with a higher yield.

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