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Challenges of SSF Process for Pea and Wheat Bran Valorization Using Trichoderma Spp. for Biocontrol Agent Production

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Abstract. **Fungal strain *Trichoderma spp.* is a known bio control agent (BCA), which are difficult to obtain using more prevalent industrial-scale biofermentation methods such as submerged fermentation (SmF) However, difficult up-scalable solid-state fermentation (SSF) are appropriate for such BCA production. An automated semi-rotating drum bioreactor was developed and built to explore SSF scale-up using pea and wheat bran as substrates to valorise food-industry by-products into viable BCA 's. Estimation of biomass using CO_2/O_2 analysis in exhaust gasses revealed that mix of pea and wheat bran can produce a viable BCA 's using gentle mixing technique with possibilities of industrial scale-up.**

Keywords: Biocontrol agent (BCA), Food waste valorisation, Solid state fermentation (SSF), Trichoderma.

I. INTRODUCTION

In recent years biopesticides have been seen as an alternative to their agrochemical pesticide counterpart and are being increasingly studied for their beneficial properties of leaving no harmful residue, which enters the food chain and reduction of environmental impact as dangerous waste water runoff [1]–[3].

When comparing solid-state fermentation (SSF) to submerged fermentation (SmF) the SSF process uses less resources, energy and has a significantly lower environmental impact than SmF methods for the production of similar products and is less resource intensive in terms of process control [4], [5]. Furthermore, SSF fermentation can be utilized to valorise various food waste products and process waste such as residue from biomethane plants and carbon rich seed shells into useful biomass for agricultural industry [1], [6], [7].

In recent years SSF has been implemented in various industries including agricultural biocontrol agents (BCA) and has a potential to produce a viable biopesticide with an extended shelf life when comparing to the same process being carried out in SmF. Some microorganisms such as *Trichoderma spp.* cannot produce viable antifungal properties within SmF cultivations due to shear forces being exerted in hyphae and lack of free oxygen [8]–[11].

However, SSF fermentation lacks the precise process controls which are available in SmF processes such as on-line O_2, pH, optical density (OD) measurements within the substrate, therefor the process is less controllable as the substrate pH and O_2 cannot be effectively controlled during the fermentation as local pH adjusting solutions (such as H_2SO_4 and NaOH) volume can 't be effectively estimated. Furthermore, heat transfer and fermentation substrate itself is heterogeneous which manifests itself as significant temperature and moisture gradient within the substrate which in turn often can lead to viability loss or death of the microorganism due to overheating and/or lack of moisture, which lowers the water activity below the threshold where microorganisms can survive [11]–[15].

Trichoderma spp. is a free-living fungi species and it has been proven to be effective BCA against various plant diseases by releasing fungi toxic substances to inhibit the growth of fungal pathogens which inhibit agricultural plant growth and diminish production potential. With biopesticides becoming more popular their production is

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still a going concern [16]–[18]. Various studies have shown that *Trichoderma spp.* exhibits a greater biomass growth and greater production of several extracellular products when the growth is undertaken in SSF in comparison to SmF. In SmF *Trichoderma spp.* doesn't exhibit significant antifungal properties only the liquid surface layer is considered to be viable BCA where free O_2 is plentiful for *Trichoderma spp.* to produce hyphae [19]–[21].

Although several substrate variations have been researched for the purpose of *Trichoderma spp.* cultivation, there is not a unified standard for food waste and utilizing food waste is challenging as the constituents are widely variable between geographical locations and food industries [7], [22], [23]. SSF imposes several challenges when process scale-up is performed in comparison to laboratory experiments such as high number of microbial variant development, large scale inoculum development as SSF requires generally higher level of inoculum compared to SmF, medium sterilization, aeration is impeded as medium tends to cake together and forced aeration takes the path of least resistance through heterogeneous system, agitation is impeded, heat removal as microorganisms excrete heat with metabolic activity, solid moisture content and water activity may decrease below sustainable threshold, pH control is impractical, heterogeneity of the medium is high, difficult downstream processing, waste management of exhaust gases and solid handling is generally more difficult in comparison to liquid handling in SmF processes [21], [24]–[29].

II. Materials and Methods

A. Microorganism and substrate parameter identification

The study was conducted with microorganism *Trichoderma asperellum* (MSCL 309) initially obtained from local soil. Initially wheat bran and pea bran were heated to 105°C for 7 days to determine the free water content in which the bran initially arrives from the production plant. Each sample was weighted before and after the drying process. Properties of each substrate are shown below in table 1. Initially tests with petri dishes were carried out, to approximate the initial starting conditions for scale up of *Trichoderma spp.* Petri dish cultivation tests with the microorganism were carried out to determine the starting parameters for SSF cultivation experiments. Each petri plate was filled with 6 grams of substrate (two with only what bran or pea bran and two with 50/50 mix with three sets for each experiment). Predetermined amount of distilled water (pH 5,6 and 7) was added to each plate twice throughout the cultivation (10, 15 and 20ml) to determine the optimal moisture content of substrate. The plates were inoculated with 10ml of *Trichoderma spp.* with an optical density (OD) of 1 at 540nm. In the last repetition inoculant was mixed thoroughly in the substrate to determine the growth characteristics of mixed vs unmixed samples. Incubation was performed in 22°C and 28°C for 168 hours. The growth activity was determined visually after the experiment and cross-compared to other samples.

B. Bioreactor and controls

Based on petri dish experiments Raimbault column type static bed laboratory bioreactor was built to perform small scale tests of SSF processes. Schematic of the preliminary tests is shown in Fig. 1 where air flow and air humidity are constant throughout the entire fermentation process and the flow was set for all preliminary tests 84 to 2 g/l*min^{-1} (calculated depending on the mass of dry substrate in the reactor) with Cole-Parmer 150mm flowmeter (type - GY-3219-19). Reactor air inlet humidity was supplemented by sparger tube inline of the reactor air inlet and was at the saturation point for air (0.017 kg/m^3) to achieve highest possible water activity without flooding the substrate. All preliminary tests within Raimbault type bioreactor were conducted for 168 hours.

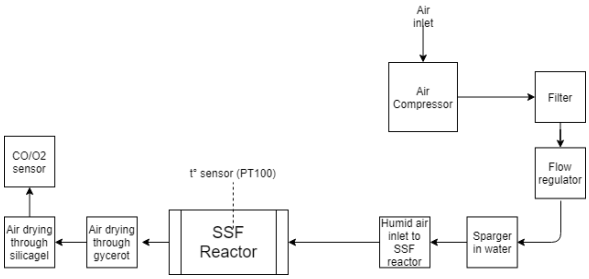

Fig. 1. Schematic of the preliminary tests is shown: Air is pumped from environment, filtered and flow is preset with flowmeter. Air is pumped through SSF reactor where it is passed from below and is exhausted through drying agents Exhausted air is ported through CO_2/O_2 sensor.

After the preliminary tests prototype for SSF cultivation was constructed by A/S "Biotehniskais centrs" (Riga, Latvia) which features air preparation with thermostating and humidification capabilities, semi-rotating drum with mesh bottom for humid air flow to the substrate. The prototype reactor is controlled by process logic controller (PLC) Siemens Simatic-S7 which controls the revolution amount, cycle count, reactor air inlet temperature and monitors temperatures within the reactor with temperature probes (PT-100) with length 150mm on top and bottom and 300mm in centre of the vessel to determine the temperature within the substrate. Air is pressure is reduced and regulated through rotameter, where it further enters the humidification column with rashig rings for maximizing surface area of air within the column. Two loop circulation system with one loop being thermostated water which heats or cools the feed water to the reactor through tube-within-tube heat exchanger. Schematic of the prototype is visualized in Fig. 2 and prototype reactor is visible in Fig. 3.

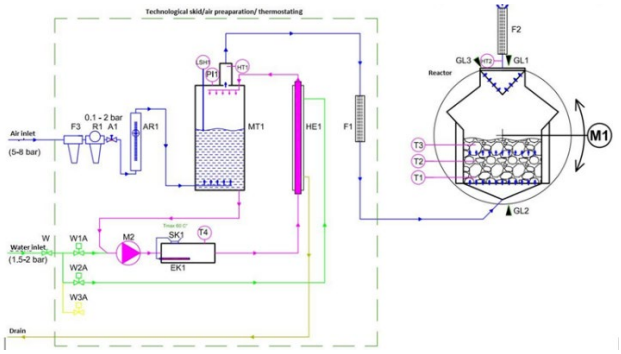

Fig. 2. Schematic representation of semi-rotating SSF reactor prototype and its principle of connections.

Fig. 3. SSF prototype reactor.

SSF cultivation of *Trichoderma spp.* was performed using wheat bran (supplied by: AS 'Rīgas dzirnavnieks') and pea bran (supplied by: SIA 'ALOJA-STARKELSEN'). The substrate required wetting and sterilization, which was performed initially just for the substrate by autoclaving the substrate at 121°C for 130 minutes, but due to overwhelming contamination issues the sterilization for later fermentations was performed for the whole vessel in the autoclave and direct steam injection on dry substrate was also explored to mitigate the contamination issues encountered during the process, experiment substrates and sterilization techniques used are overviewed in Table 1.

Fermentation process was set to run for 168 hours from the inoculation to full stop, rotation cycles were varied throughout the process with the set points for rotation count, delay of initial rotation since the process start, interval between rotation cycles and hold time when the vessel has rotated 180°.

TABLE I. TABLE 1 FERMENTATION SUBSTRATE PARAMETERS

Process No.	Pea waste (g)	Wheat bran (g)	expanded clay 0-4mm (g)	Water (g)	Autoclaved time (min)	Autoclaved temp	Moisture g/g	Moisture g/g
1	0	3,300	0	6,450	35	121	1.95	1.95
2	0	5,500	0	10,000	35	121	1.81	1.81
3	0	5,500	0	5,000	35	121	1.81	1.81
4	5,500	0	0	5,500	35	121	1.00	1.00
5	5,500	0	0	4,000	90	121	1.00	1.00
6	0	5,000	0	4,000	90	121	0.80	0.80
7	0	5,000	0	10,000	90	121	2.00	2.00
8	0	5,000	0	10,000	90	121	2.00	2.00
9	0	5,000	0	10,000	90	121	2.00	2.00
10	0	3,615	4,695	7,000	120	121	0.84	0.84
11	0	3,615	4,695	7,000	120	121	1.94	1.94
12	0	3,615	4,695	7,000	120	121	1.94	1.94
13	7,050	0	0	6,650	120	Direct steam injection	0.94	0.94
14	3,650	1,750	0	3,700	120	Direct steam injection	0.69	0.69
15	0	3,200	1,950	3,150	180	Direct steam injection	0.61	0.61

Fermentations were run both with additional humidification and without humidification to combat infection issues which might be present if the water in humidification column was contaminated. To mitigate contamination issues which might be present in the humidification system 10 grams of $KMnO_4$ was added to the water and separatory sedimentation bottle was added in line with the air food to the SSF reactor in order to catch any remnants of potassium permanganate before entering the reactor itself.

Before each experiment filters, tubes, connectors and all adjacent accessories were autoclaved at 121°C for at least 30 minutes to ensure sterility. Substrate was weighted, wetted and autoclaved in polypropylene bags and poured into vessel while hot. Before each process SSF reactor was washed, and disinfected with ethanol spray and hydrogen peroxide.

After repeated failed fermentation expanded clay was added to aid the aeration of the substrate as well ass to improve substrate drainage properties alongside with clumping issues which were observed initially. With ongoing failed attempts and contamination issues the vessel itself was autoclaved in processes No.3 and No.4. The room dedicated to the vessel was irradiated with short wave UV light to improve the sterility of the environment.

With persistent contamination issues the vessel was filled with dry substrate and steam was directly injected into the substrate at atmospheric pressure while condensing wetting the substrate and disinfecting the vessel thus lessening the operations where contamination might be introduced into the vessel.

In the start of each process when the substrate and vessel was still too hot for the microorganism it was let to cool down naturally and *Trichoderma spp.* was introduced only when substrate had cooled at least below 30°C.

III. Results And Discussion

A. Initial Trichoderma harzianum. growth specifics with variable water activity, substrate 104 composition and pH

After incubating for seven days the samples at 22 º C, it was observed that *T. harzianum* has grown in all wetted substrate samples. The highest growth activity was found in a sample containing 10 ml of distilled water, respectively a growth activity value of 4 out of 5, in a sample of 15 ml and 20 ml of water, a value of activity of 3 out of 5. It could be visually established that in samples where *T. harzianum* was not further mixed into the substrate, mycelium has grown more evenly into the substrate. *T. harzianum* grows best at 10 ml distilled water humidity 22 º C see Fig. 4.

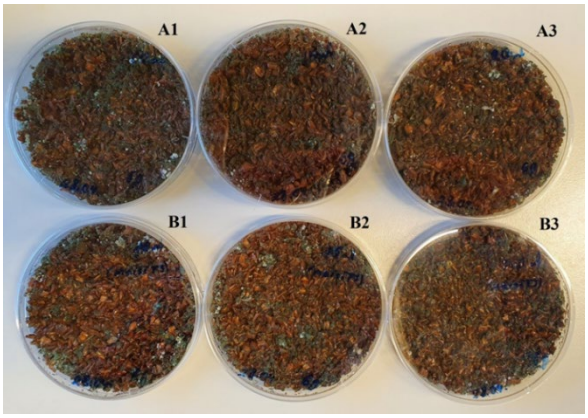

Fig. 4. T. harzianum growth activity at different humidity values. A – suspension not mixed into substrate, B-suspension mixed into substrate; 1-10 ml, 2-15 ml, 3-20 ml.

After incubating the next samples for seven days at 22 º C, it could be established that *Trichoderma harzianum*. has grown in all samples, regardless of pH values or the variety of the substrate. The highest growth activity was found in a substrate consisting of three grams of pea bran and three grams of wheat bran, and the pH of the distilled water required for humidity was 7. A growth activity value of 5 of 5 was assigned.

Samples using six grams of pea bran for growth activity could be assigned a value of 2 out of 5 as a substrate, while the best was found at pH 7. In a sample using six 120 grams of wheat bran as a substrate, *Trichoderma harzianum*. was visually different from all other samples within seven days, possibly the secondary growth characteristics began to appear, a given growth activity value could not be objectively assigned to the sample concerned, nor did the pH values differ between the samples. All samples were moistened with 10 ml of water and incubated at 22 º C see Fig. 5.

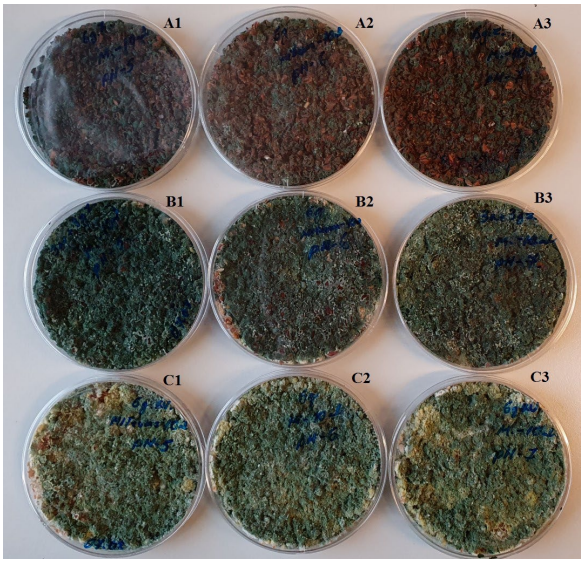

Fig. 5. T. harzianum growth activity at different pH values and different substrates. A - pea bran, B-pea bran + wheat bran, C-wheat bran; 1-pH 5, 2-pH 6, 3-pH 7.

After incubating the samples for seven days at 22°C and 28°C, it could be established that *Trichoderma harzianum*. has grown in all samples, regardless of temperature values or substrate diversity. The highest growth activity was found in a substrate composed of three grams of pea bran and three grams of wheat bran, and incubated for seven days at 22°C. For the sample concerned, a growth activity value of 5 was assigned. Samples using six grams of pea bran for growth activity could give a value of 2 out of 5 as a substrate, while the best was found at 22°C, respectively activity was 3 out of 5. All samples were moistened with 10 ml distilled water with a pH value of 7.

After the test results in petri dishes the initial parameters for SSF could be established that the best temperature to achieve is 22°C, the substrate should have a pH of 7 and substrate of pure wheat bran demonstrated the highest growth potential.

B. Initial Trichoderma harzianum. Initial scale-up and experiments in static raimbault column type bioreactor

SSF processes were evaluated in smaller scale static column repurposed and rebuilt submerged cultivation bioreactor (4L) with inlet water humidification and CO_2/O_2 exhaust gas analyser. However, the scale up solution includes a custom-built prototype with an intermittently rotating drum, which has the internal shape of a uniform cube to mitigate the effects of preferential path development during the substrate forced aeration processes.

Relative humidity in the delivered substrate constituents was determined by drying five samples of approx. 5g in weight over three days in a vented oven with internal temperature control setpoint of around 105 °C for 70h each. The drop in weight was accounted as water separation through evaporation over time. The same amount (5 grams) of the same substrate from the same batch of substrate was soaked in water over 24h and the swollen substrate was then placed on sieve and allowed to separate of all the excess water until there is no dripping observed and the maximum water absorption was determined by weight gain. Relative humidity and maximum non-drip water absorption were determined for both substrates, with wheat bran produced delivered humidity determined to be at W.

During the SSF fermentation the lag phase was observed to be around 22 hours from the inoculation of the substrate and exponential growth was observed until 64 hours regardless of the substrate. Wheat bran with initial moisture of 65%.

After the fermentation the wheat bran substrate had interwoven with fungal strands (see Fig. 6) and was difficult to extract from the reactor as the whole substrate had transformed from loose mass to a uniform piece. Coffee bran substrate did not encounter the similar problems as the substrate was loose even after the fermentation.

Fig. 6. T. harzianum Trichoderma SSF fermentation in wheat bran in static raimbault type column from left to right - 0h, 64h, 168h.

C. Fermentation in SSF prototype reactor

The experimental procedure is aimed at achieving the best possible results and each process parameters are evaluated continuously. The initial process parameters are based on the preliminary tests and are scaled-up based on the weight of the substrate in the reactor. Recorded data from probes T1, T2, T3, HT1, HT2 were monitored continuously and logged within the PLC unit of the SSF prototype bioreactor.

The fermentation results were variable and the rotation of the vessel in combination with wetted substrate introduced clumping issues which were further exacerbated by the fungal hyphae growth. First batch of SSF prototype with wheat bran was unsuccessful due to contamination and the process had failed. Improved methods for SSF reactor preparation had been devised and the procedure was reviewed to make a successful fermentation using *Trichoderma spp.*

The second fermentation with wheat bran and the parameters shown in Table 3.2 was successful and the process was carried out for 104 hours. Results from the sensors are shown in Fig. 7, which correspond to the sensors shown in Fig. 2.

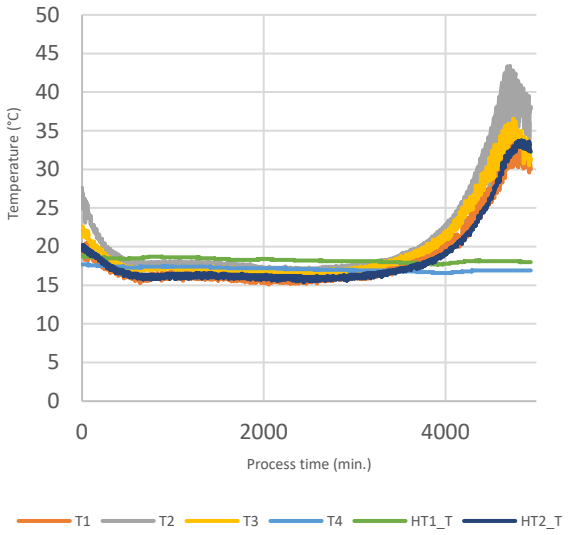

Fig. 7. SSF prototybe bioreactor sensor data from SSF fermentation No2.

From the process temperatures it is clearly visible, that every rotation cycle has a temporary decrease of temperature within the bioreactor, however the sensos T2, which is in the centre within the substrate recorded a maximum temperature if 43°C, which is significantly higher than the temperature for sustainable *Trichoderma spp.* Growth and inhibits the process.

The lag phase of the process is quite long (approx. 3000 minutes) when no activity can be measured by temperature increase, however temperature increase is not representative of the biological activity as the heat generated by biological activity was lower before the 3000-minute mark because the excess heat was exhausted with humid aeration gasses. Exhaust gas O_2/CO_2 content analysis should be performed to determine the lag phase properly.

Fig. 8. Wheat bran directly after fermentation.

After fermentation wheat bran had formed various size agglomerates with hyphae stands as their structure elements and being held together by them. These agglomerates are undesirable within the SSF reactor as they inhibit the air circulation within them and introduce a path of least resistance for air to travel through. After leaving the product in the room and covered with plastic wrap, it developed typical fungi fuss and exhibited significant biological activity (Fig. 9).

Fig. 9. SSF fermentation product from process No.2 after a week of storage.

The reproduction of the processes in pilot scale semi rotating drum bioreactor were unsuccessful, the experiments were not successful as the unknown contamination (suspected contamination from bacillus processes) was overwhelming the fermenting vessel while Trichoderma was outcompeted by the contaminants within the vessel.

During the experiments there were numerous approaches and attempts to eliminate the contamination source which included and were performed in various combinations.

- Disinfection of the room with 70% ethanol solution including outer exterior of the vessel, walls, ceilings, floor etc.
- Irradiating the whole room with disinfecting UV-Ray lamps
- Disassembling the whole reactor vessel and autoclaving the vessel with wetted substrate in autoclave at 121°C for up to 120 minutes
- Disinfection and flushing of the whole humidification column with fresh water and hydrogen peroxide
- Adding potassium permanganate to humidification column as a disinfectant to assure that no foreign microorganisms could enter the humidification column
- Performing the SSF fermentation experiments without usage of humidification as to eliminate humidification columns as a source for infection
- Before each process all filters, tubes and any removable hardware was autoclaved separately at 121°C for 30 minutes and installed upon bioreactor only when substrate was added

- Disassembling the barrel from the skid as and performing direct steam injection at atmospheric pressure for at least 120 minutes
- Replacing the silicone seals before the process as a precaution to eliminate possible source of infection which has remained in potentially micro ruptures in the seals
- Changing the media from wheat bran and pea bran to whole rye grain, which had a limited success as to rule out contaminant possibility from the initial substrate

All the processes had very heterogenous structures, where the top layer and bottom layers had the most microbial activity, although it is hard to draw conclusions as many of the processes were contaminated and repeatability was very low.

Temperatures were usually higher than a maximum permissible temperature of 35°C, (see Fig. 10) which is significantly higher than the temperature for sustainable *Trichoderma spp.* Growth and inhibits the process, it is clear from the process temperatures that every rotation cycle has a temporary decrease of temperature within the bioreactor.

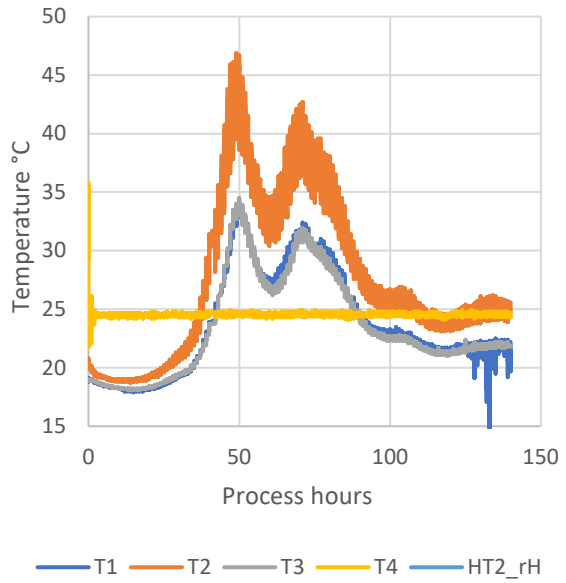

Fig. 10. Process No.15 of unsuccessful, contaminated fermentation.

IV. Conclusions

SSF fermentation is a challenging process, where several limiting factors are to be taken into account, which usually are mutually conflictive and the production of optimum environment for *Trichoderma spp.* cultivation is difficult to achieve.

The prototype reactor itself served the purpose as initially planned and only minor technical difficulties were encountered such as plugged filters and pumping inefficiencies in humidification column, however the scale-up technology for cultivation of BCA`s the reactor design is not suitable due to numerous problems associated with continuous infection within the reactor by an unknown microorganism.

For scale-up purposes SSF continues to be labour intensive with difficulties of setting up the process, autoclaving the substrate, vessel and accessories as well as manual labour involved when the reactor is discharged and cleaned for preparation of the next process, which has to be done manually while in SmF type reactors the operations are significantly less labour intensive due to established CIP (Clean in place) and SIP (sterilisation in place) procedures as well the fact that the substrate within the vessel is easily transferred with various types of pumps.

To achieve successful SSF processes it is suggested that the prototype reactor should be designed with vastly different assumptions of SSF process as rotation of the reactor itself introduced variable voids and clumps within the substrate itself where water activity was low the aeration was high, as well as dead zones where no aeration was observed it led to anaerobic fermentation conditions.

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