



Recycling of cell culture and efficient release of intracellular fructosyltransferase by ultrasonication for the production of fructooligosaccharides

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ABSTRACT

Production of fructooligosaccharide (FOS) through efficient cultivation of biotransformation process by fructosyltransferase (FTase) was evaluated by two new isolates, *Aspergillus niger* and *Aspergillus flavus* NFCCI 2364. The saccharide consumption revealed lag phase of *A. niger* in 10 h which were smaller extent than *A. flavus* of 14 h. For the recycling of cell culture, the pellet cells were continuously reused after 24 h of submerged fermentation by these microorganisms in which FTase activity remains stable in four consecutive cycles in *A. niger* and six cycles in *A. flavus*. When freshly prepared pellets were sonicated for efficient release of intracellular FTase, the best transformation reaction was performed at 20 W acoustic power giving conversion yield of FOS 61.43% (w/w) by *A. niger* and 70.44% (w/w) by *A. flavus* respectively. This study was shown that the two fungal isolates can serve as veritable source of intracellular FTase for industrial production of FOS.

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1. Introduction

Fructooligosaccharide an important food candidate with several functional benefits exposes new perspectives in nutrition and food sciences from the last two decades. Fructooligosaccharides (FOS) are oligosaccharides of fructose consisting of one to three fructose units bound by β 2 $\rightarrow$ 1 position of sucrose (Maugeri & Hernalsteens, 2007). FOS represent major group of fructan oligomers and are mainly composed of 1-kestose (GF2), nystose (GF3) and 1-fructofuranosylnystose (GF4). They are considered both alimentary additives and nutraceutical that are not susceptible to decomposition by human or animal digestive enzymes (Mussatto, Rodrigues, & Teixeira, 2009; Yun, 1996). Due to its various interesting functionalities the market potential of FOS further includes applications in pharmaceutical and diagnostic sector underlying great industrial value (Fernandez, Ottoni, Silva, Matsubra, & Carter, 2007; Maiorano, Piccoli, da Silva, & de Andrade, 2008; Zuccaro, Gotze, Kneip, & Dersch Seibel, 2008; Ganaie et al., 2014). The important health benefits include bifidogenic property, bioavailability of minerals, non-cariogenic property, safe for diabetes and prevention of colonic carcinogenesis (Dominguez

et al., 2012; Ganaie, Lateef, & Gupta 2014; Sangeetha, Ramesh, & Prapulla, 2004). The synthetic process of FOS is carried out by transfructosylating process of fructosyltransferase (FTase) or β -fructofuranosidase (FFase) from many plants, bacteria and fungi (Ganaie, Gupta, & Kango, 2013; Mussatto et al., 2012). The FOS producing enzymes from microorganisms are excreted either outside of cell as extracellular enzyme or retained within the cell as an intracellular enzyme. A variety of methods have been used to break up cells and each one having its advantage and disadvantage (Chisti & Young, 1986). Ultrasonication a liquid shear method has received much attention for disruption of microbial cells in suspension at laboratory scale. This method is suitably used for industrial purpose, since it provides low operational cost, neither sophisticated device nor extensive technical skills (Barton, Bullock, & Weir, 1996; Feliu, Cubarsi, & Villaverde, 1998; Ozbek & Ulgen, 2000). Ultrasound in enzyme solutions leads positive impact on enzyme activity. It increases growth of microorganisms but high frequency ultrasound of 15–20 kHz is capable of causing disruption of cell wall thereby releasing intracellular enzymes (Matsuura, Hirotsune, Nunokawa, Satoh, & Honda, 1994; Swamy, Sukla, Narayana, Kar, & Panchanadikar, 1993), while too high intensity ultrasound (>20 kHz) can cause denaturation of enzyme (Mason, Paniwink, & Lorimer, 1996). Ultrasonication had been widely used for obtaining various intracellular enzymes from microbial cells in order to release enzymes like glucose oxidase from *Aspergillus niger* CCT 7415 (Ishimori, Karube, & Suzuki, 1982), β -galactosidase of *E. coli*

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and *Lactobacillus* strain (Feliu et al., 1998; Wang & Sakakibara, 1997), invertase from *Aspergillus niger* (Vargas, Piao, Domingos, & Carmona, 2004) and fructosyltransferase from *Aureobasidium pullulans* CFR 77 (Lateef, Oloke, & Prapulla, 2006) respectively. The mechanism of cell disruption by ultrasound is due to cavitation phenomenon in which microbubbles form at various nucleation sites in fluid. These bubbles grow during refraction phase of sound waves, and then in compression phase, these bubbles collapse releasing violent shock wave which propagates through the medium. Through this phenomenon, large quantity of sonic energy is converted into mechanical energy in form of elastic waves (Chisti & Young, 1986).

In our previous study, *A. flavus* (NFCCI 2364) and *A. niger* (SI) proved to be potential producers of FOS (Ganaie, Gupta et al., 2013). These strains also produced compact fungal pellets when grown under submerged fermentation. The main objective of present work is to investigate effect of ultrasound on release of intracellular FTase from these strains and its impact on FTase activity for FOS production. For successful performance of FOS yield, recycling of cell culture was also investigated so as to avoid the need for the development of fresh inoculum.

2. Materials and method

2.1. Microorganisms and culture conditions

A. niger (SI), a soil isolate microorganism obtained from culture collection Department of Applied Microbiology and Biotechnology, Dr. H.S. Gour University Sagar and *A. flavus* NFCCI 2364 obtained from Culture Collection ARI, Pune, India were used in this study. Both these microorganisms were grown on potato dextrose agar (PDA) at 28 °C and were kept on slants at 4 °C until further use (Supplementary Figs. S1 and S2). Cultivation of FTase was performed in 250 ml shake flask containing sucrose 20%, yeast extract 0.5%, NaNO₃ 1%, MgSO₄·7H₂O 0.05%, KH₂PO₄ 0.25%, NH₄Cl 0.5%, and NaCl 0.25% in 100 ml of distilled water with an initial pH of 6.0. The medium was sterilized at 110 °C for 15 min and incubated on rotary shaker (Lark Germany) at 28 °C for 72 h. At the end of respective fermentation periods, flasks were withdrawn at regular time intervals and their contents were filtered through Whatman filter paper No. 2, 110 mm diameter. The cell free culture filtrate was used as a source of extracellular enzyme without further purification. All the experiments were carried out in triplicate for determining FOS formation comparing with its standard components 1-kestose, 1-nystose, 1-β-fructofuranosylnystose and sugars such as sucrose, glucose, fructose from Wako Chemical Laboratories (Japan) and Sigma–Aldrich (USA) respectively (Supplementary Figs. S3 and S4).

2.2. Recycling of cell culture

For recycling, the 24 h pellet cells were separated by decanting the culture broth into container in aseptic conditions and fresh cultivation media was added. At the end of every next 24 h, broth was decanted and fresh media was added consecutively up to seven cycles (168 h). The fermented decanted broth was investigated for change in pH, transfructosylating activity and FOS formation.

2.3. Extraction of FTase enzyme by ultrasonication

After 72 h of fermentation, the freshly prepared harvested media of both microorganism *A. niger*, and *A. flavus* were centrifuged at 4 °C in cooling centrifuge (Eltek RC 8100 SF, India) and compact pellet cells were resuspended in 100 ml of cold distilled water for ultrasonication to obtain intracellular FTase. Ultrasonication of cells was carried out using Ultrasonic processor 200 W (Soniweld, Imeco, Ultrasonics, Mumbai, India). A probe type wave guide 2 mm diameter was immersed at a depth of 3 cm into 20 ml of cell suspension

and two level of acoustic power 20 W and 40 W were investigated for irradiation period of 2–12 min. The sonication was done at 10 °C and cell suspension was kept in salt ice bath during disruption to prevent over heating. The ultrasonic energy was pulsed 0.5 s active and passive intervals for reduction of free radical formation (Lateef et al., 2007). At the end of ultrasonication, cell free lysate was centrifuged at 4 °C and crude FTase was used as source of intracellular enzyme for FOS production. The release of FTase enzyme was monitored by determining OD at 275 nm and protein content of the fraction by the Bradford method.

2.4. Enzyme assay

The FTase activity was determined by incubating 250 μl of enzyme with 750 μl of sucrose 60% (w/v) in 0.1 M citrate buffer (pH 6.0) at 55 °C for 1 h in water bath. At the end, reaction was stopped by keeping the reaction mixture in hot boiling water bath up to 10 min. Transfructosylating activity (Ut) was done by diluting 20 μl of reaction mixture with 980 μl of distilled water in order to make 50-fold dilution. Then, 10 μl from 1 ml reaction mixture was allowed to react with 1 ml test reagent using glucose oxidase peroxidase kit (Sigma) for 30 min until pink colour develops. The absorbance of glucose released was read at 505 nm on UV/visible spectrophotometer (Hitachi Techcom India). The units of fructosyltransferase (Ut) and hydrolytic (Uh) activities were defined as the amount of enzyme that required to release 1 μmol of glucose per ml per minute under the chosen experimental conditions.

2.5. Production and analysis of fructooligosaccharides

FOS production was carried out using reaction mixture of FTase and sucrose of (1:3) ratio in shaker incubator (Lark Innovata Germany) at 55 °C for 24 h. At the end of incubation reaction was arrested in boiling water bath at 100 °C for 10 min. The samples were diluted and filtered through membrane filter millipore 0.45 μm pore size and analysis was done by HPLC at room temperature 30 °C. The HPLC instrument was from Waters (USA) with RI detector 2414 and injection valve of 20 μl. Sugar pak column (6.5 × 300 mm) was used for identification and quantification of FOS derivatives kestose (GF₂), nystose (GF₃), 1-β-fructofuranosyl nystose (GF₄) and sugars such as sucrose (GF), glucose (G), fructose (F) respectively. The column temperature was kept 70 °C and RI detector temperature at 30 °C. The solvent system used was water as mobile phase at flow rate of 0.2 ml/min. Calculation of analysis was done by Empower 2 software Build 2154 Waters (USA).

2.6. Statistical analyses

The data in the tables and figures were expressed in triplicates as mean ± standard deviation. Using one-way analysis of variance (ANOVA), a difference was considered statistically significant if the *p* value was less than 0.05.

3. Results and discussion

3.1. Consumption and production of saccharides in cultivation media

After spore inoculation in cultivation media the selected microorganisms showed disparate growth pattern. The concentration of saccharides formation during course of fermentation periods was analyzed by HPLC. The results showed that the lag phase of *A. niger* was 10 h which was comparatively less as compared to *A. flavus* whose lag phase stayed up to 14 h before FOS formation commence. The lag phase of these microorganisms were quite

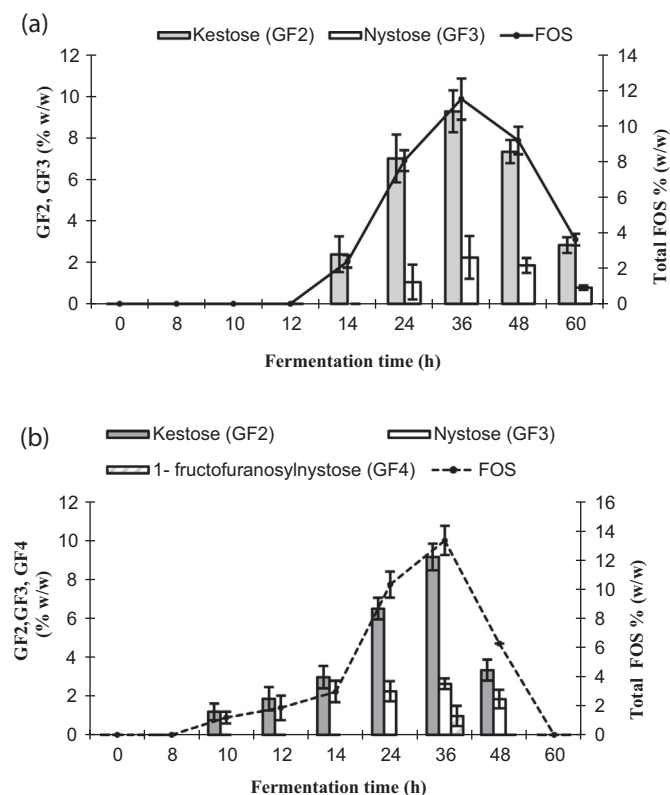


Fig. 1. FOS production of *A. niger* (a) and *A. flavus* (b) using shake flask cultures during 60 h of cultivation period. The data is represented by means of triplicates experiments with standard deviation.

different compared to those earlier reported for other FTase producing microorganisms like *Penicillium expansum* and *Aspergillus japonicus* whose lag phase remained up to 12 h (Mussatto et al., 2009; Prata, Mussatto, Rodrigues, & Teixeira, 2010). After exit of lag phase, these microorganisms consumed saccharides for growth and partly for FOS formation. In their growth configurations there occur some morphological changes due to increase in intracellular contents which maximize the cell growth rate. Since sucrose was almost depleted within 36 h of growth from both these microorganisms and concentration of FOS starts to decline, which stated that the microorganisms have been consumed FOS for their growth and survival (Supplementary Figs. S5 and S6). The maximum amount of FOS formation by *A. niger* was observed 13.37% (w/w) in which concentration of kestose was remarked 9.18% (w/w), nystose 2.64% (w/w), 1-fructofuranosylnystose 0.97% (w/w) respectively (Fig. 1a). In contrast to *A. flavus*, there was no formation of 1-fructofuranosylnystose (GF₄) during whole fermentation process, and the total FOS content was observed 11.52% (w/w) (Fig. 1b).

FOS formation is a two stage experimental process. The first stage refers to the production of FTase from microorganisms, and in the second stage, FTase is employed for building of FOS oligomers in enzyme substrate reaction (Yun, 1996). During fermentation the two processes occur simultaneously, leading to less amount of FOS formation which is not suitable for an industrial purpose. In addition, during fermentation, fungi usually grow best at 28–30 °C, whereas FTase show high transformation activity up to 50–60 °C (Barthomeuf & Pourrat, 1995; Lateef et al., 2008). All these events ultimately lead to poor yield of FOS.

3.2. Continuous production of FOS by recycling of cell culture

Improvement in productivity of FTase was employed by recycling of cell culture by *A. flavus* and *A. niger* in a medium

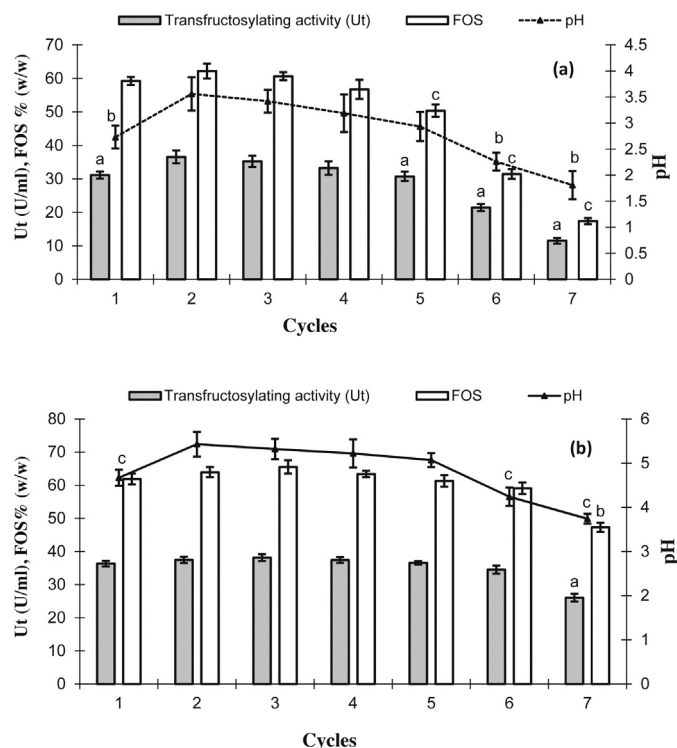


Fig. 2. Production of fructooligosaccharides by recycling cell culture of *A. niger* (a) and *A. flavus* (b). a, b, c represent statistically different from each other batch so noted ($p < 0.05$) using one-way ANOVA followed by Tukey's post test.

containing sucrose as a sole carbon source. After 48 h of fermentation in *A. niger*, an increase in FTase activity was observed from the 2nd cycle (36.57 U/ml) which remained almost constant up to the 4th cycle. Soon after this, significant decrease ($p < 0.05$) in enzyme activity was observed in subsequent cycles (Fig. 2a). Highest FOS yield of 62.2% (w/w) was observed from 2nd cultivable cycle and this value was almost maintained up to 4th cycle at an average of $59.71 \pm 2.32\%$ (w/w). A drastic fall of pH within 48 h fermentation was observed in *A. niger* and 4.67 in *A. flavus* respectively which intensified on next cycles due to replenishment of fresh media. The pH tends to decrease significantly ($p < 0.05$) from both microorganisms after 5th cycle possibly due to the release of some organic acids by disintegration of reused pellets. In contrast to *A. flavus* cell culture, highest transfructosylating activity (Ut) was found in the 3rd recycle (38.14 U/ml) and this value was almost sustained in consecutive six cycles at an average of 36.74 U/ml. FOS formation was consistently harvested up to six cycles at an average of 62.25 ± 2.26 contributed to maximum concentration at 3rd cycle $65.53 \pm 1.01\%$ (w/w) which significantly decreased ($p < 0.05$) after 6th cycle (Fig. 2b).

The results of our experimental studies are comparatively greater by Sangeetha, Ramesh, and Prapulla (2005) which maintained 15 U/ml of FTase up to six cycles and harvested 53% (w/w) of FOS using recycling culture of *Aspergillus oryzae* CFR 202. Furthermore, Mussatto et al. (2009) reported constant β -fructofuranosidase activity 40.6 U/ml by immobilized cells in seven cycles which further decreased by 22% at the eighth cycle. However in their experimental studies FOS concentration was maximum 134.6 g/l in first cycle which progressively decreased after successive cycles. Recycling of cell culture is considerably beneficial for an industrial purpose, because FTase are retained without need of any supplementation in media and also avoidance of repeated inoculum preparation.

Table 1
Effect of ultrasonication on the release of total protein content, transfructosylating activity (Ut) and FOS production by intracellular enzyme of *A. niger* at 20 W and 40 W acoustic powers.

Time (min)	20 W (acoustic power)			40 W (acoustic power)		
	(Ut) U/ml	Protein content (μg/ml)	FOS % (w/w)	(Ut) U/ml	Protein content (μg/ml)	FOS % (w/w)
2	10.28 ± 1.34 ^a	20.22 ± 1.08 ^b	36.46 ± 0.80 ^c	17.51 ± 0.80 ^d	32.43 ± 0.68 ^e	25.87 ± 0.73 ^f
4	26.84 ± 1.10 ^a	26.70 ± 1.17 ^b	48.27 ± 1.28 ^c	26.17 ± 0.35 ^d	37.15 ± 0.21 ^e	32.18 ± 1.22
6	36.49 ± 0.94	32.85 ± 1.01 ^b	61.43 ± 0.94	16.90 ± 0.55 ^d	43.38 ± 0.73 ^e	21.25 ± 1.32 ^f
8	18.63 ± 0.75 ^a	37.57 ± 0.71 ^b	41.21 ± 0.92 ^c	11.07 ± 0.91	47.38 ± 1.07 ^e	15.27 ± 1.06 ^f
10	15.62 ± 1.23 ^a	43.33 ± 1.06	26.41 ± 0.61 ^c	8.51 ± 0.75	51.85 ± 1.23	9.38 ± 0.62 ^f
12	11.58 ± 0.60 ^a	47.28 ± 1.10	21.54 ± 0.93 ^c	5.82 ± 0.80	52.64 ± 1.27	5.76 ± 1.07 ^f

^{a b c d e f} represent statically significant difference ($p < 0.001$) of highest comparable value using one-way ANOVA followed by Tukey's post test.

Table 2
Releasing of protein content and transfructosylating activity of FOS production by ultrasonication of *A. flavus*.

Time (min)	20 W (acoustic power)			40 W (acoustic power)		
	(Ut) U/ml	Protein content (μg/ml)	FOS % (w/w)	(Ut) U/ml	Protein content (μg/ml)	FOS % (w/w)
2	14.65 ± 0.80 ^a	12.81 ± 1.18 ^b	32.49 ± 0.52 ^c	24.59 ± 1.34 ^d	25.79 ± 1.09 ^e	34.31 ± 0.92
4	26.70 ± 0.55 ^a	18.62 ± 0.71 ^b	38.23 ± 0.84 ^c	30.39 ± 1.25	31.90 ± 1.53 ^e	40.37 ± 0.68
6	33.68 ± 0.92 ^a	25.87 ± 0.71 ^b	56.35 ± 0.97 ^c	26.55 ± 0.99	36.40 ± 0.79 ^e	36.75 ± 1.15
8	37.51 ± 1.11	30.23 ± 0.46 ^b	63.75 ± 0.81 ^c	23.60 ± 1.33	41.67 ± 0.90 ^e	21.72 ± 0.89 ^f
10	40.31 ± 1.05	33.93 ± 1.06 ^b	70.11 ± 0.77	17.41 ± 1.34 ^d	46.78 ± 0.94	15.23 ± 1.22 ^f
12	32.19 ± 1.79 ^a	40.01 ± 0.48	61.06 ± 0.46 ^c	11.32 ± 0.57 ^d	49.91 ± 1.52	11.45 ± 0.97 ^f

^{a b c d e f} represent statically significant difference ($p < 0.001$) of highest comparable value using one-way ANOVA followed by Tukey's post test.

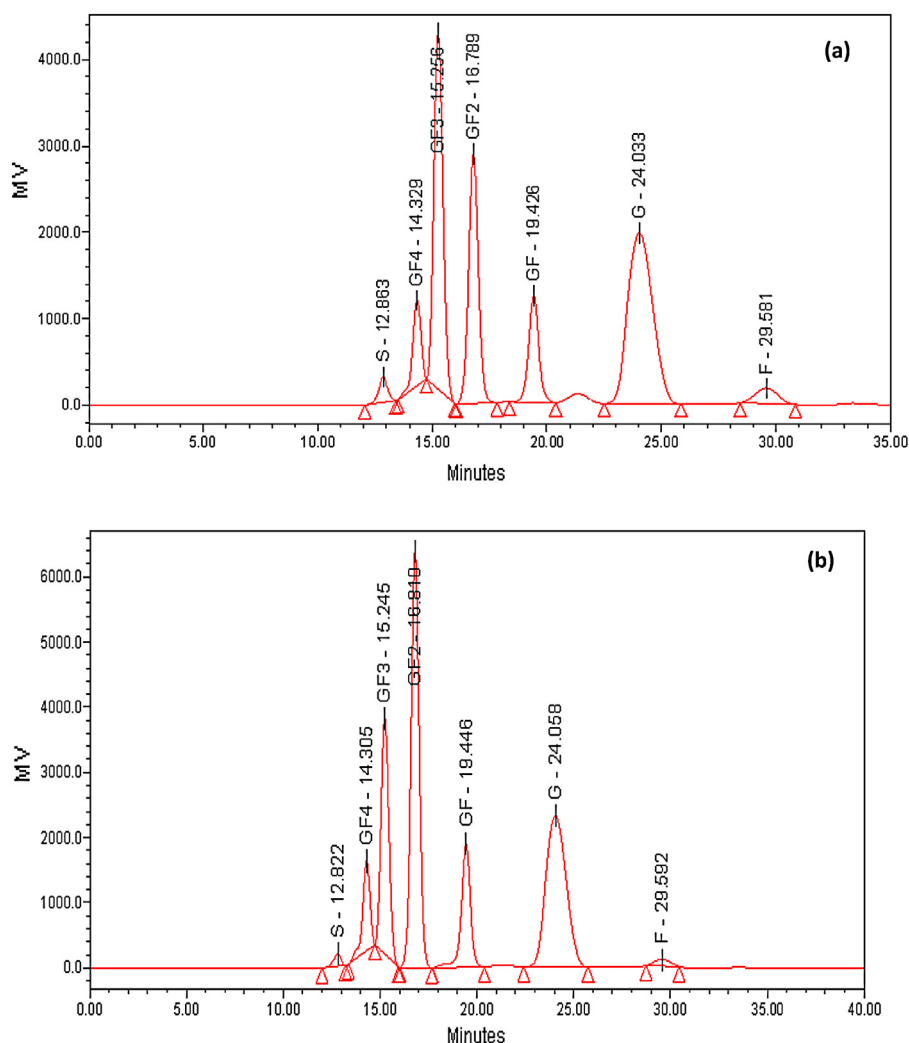


Fig. 3. HPLC chromatogram of FOS formation by intracellular FTase of *A. niger* (a) and *A. flavus* (b). (S = solvent, GF4 = 1-β fructofuranosylnystose, GF3 = nystose, GF2 = kestose, GF = sucrose, G = glucose, F = fructose).

3.3. Efficient release of intracellular FTase by ultrasonication

The efficient release of intracellular FTase from *A. niger* and *A. flavus* were executed by ultrasonication. Release of enzyme was done by two acoustic powers (W) 20 kHz and 40 kHz with processing time of 2–12 min. The amount of protein content increased as processing time and sonic energy was intensified. The ambient pressure on cell suspension led to cell disintegration and this treatment where it lasted longer than 4 min caused enzyme activation in addition to release of the cell components. The energy acted directly on the biomolecules for disrupting the pellets and later it acted in three-dimensional structure of the enzyme, thus increasing the FTase activity. Maximum FTase activity was found at 20 W acoustic power with processing time of 6 min in *A. niger* and 10 min in *A. flavus*. There after, the FTase activity decreased significantly ($p < 0.001$) in other processing times (Tables 1 and 2). The prolongation of processing time of sonication admittedly decreased the FTase activity due to release of inhibitory proteins and existence of lower thermal stress. The amount of FOS formation from intracellular FTase in *A. niger* marked 61.43% (w/w) in which concentration of nystose 32.11% (w/w) was greater as compared to kestose 25.02% (w/w) and 1-fructofuranosylnystose 4.3% (Fig. 3a) due to disproportionate type reaction mechanism. However in *A. flavus*, the amount of FOS formation was observed as 70.11% (w/w) which is the highest FOS yield by any known microorganisms. Because only 3% of sucrose was accumulated in reaction mixture and rest was transferred into formation of FOS oligomers like kestose (40.12%) nystose (24.22%), 1-fructofuranosylnystose (5.77%) in 24 h of enzyme substrate reaction time (Fig. 3b).

Least transfructosylating activity of FOS formation was found in 40 W acoustic power due to release of other inhibitory proteins which affected the enzyme activity. Thus, low ultrasound irradiation 20 W acoustic power proved to be appropriate method for releasing of intracellular FTase enzyme though disruption of hyphae. This is an interesting result as pellets of *A. flavus* were slightly compact as compared to *A. niger* which were soft and pliable and disintegrates quickly. The cell wall of *Aspergillus* sp. usually contains 10–20% chitin which had an enormous tensile strength and contributes to the integrity of cell wall (Bowman & Free, 2006). The release of proteins and enzymes is based on their molecular weight pattern. For instance alkaline phosphatase is located in cell membrane and they are released slowly during cell disruption where as presence of alcohol dehydrogenase in cytoplasm occurs in a particular moment (Melendres, Honda, Shiragani, & Unno, 1993; Wagner, Marc, & Engasser, 1992). In a study carried out by Lateef et al. (2007) FOS yield of 59% was obtained using intracellular FTase of *Aureobasidium pullulans* CFR 77 released through sonication at acoustic power of 20 W within 9 min. Earlier report of Vargas et al. (2004) marked 20–40 kHz as being adequate for the release of intracellular invertase from *A. niger* CCT 7419. Maximum release of invertase in molasses medium supplemented with peptone was achieved at irradiation of 20 kHz for 8 min.

4. Conclusion

This work has demonstrated that both fungal isolates used in this study have great ability for biotransformation of sucrose to FOS. Due to their rapid growth in fermentation media, they produce high titres of FTase, which could be applied for better production of FOS. By using recycle cell cultures, the progressive production of FOS was achieved, which can ensure avoidance of regular inoculum development. Through ultrasonication, efficient release of intracellular FTase of these fungal strains was demonstrated, leading to high formation of FOS. Finally, it can be concluded that these microorganisms are powerful producers of FOS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.carbpol.2014.03.066>.

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