Chapter 16
Nanocellulose Production Using Cellulose Degrading Fungi

Nadanathangam Vigneshwaran and Prasad Satyamurthy

Abstract Nanocellulose, a novel material derived from cellulosic biomass, consists of cellulose having at least one dimension in the nano-size (<100 nm). Very high surface area to volume ratio (50–200 m^2/g), high tensile strength (1–10 GPa) and low density (1.45 g/cc) make nanocellulose an attractive material as reinforcement agents in high performance composites. Earlier, nanocellulose was produced by concentrated sulphuric acid hydrolysis that removed the amorphous region leaving behind highly crystalline nanocellulose whiskers. Though they are stable due to sulfation on surface, scaling up could not be achieved due to reasons related to handling of concentrated (64 %) sulphuric acid and effluent disposal. Recently, research effort is towards mechanical preparation of nanocellulose by high pressure homogenization process that could circumvent the effluent problem. But, here the bottleneck is very high energy consumption (30,000 kWh/tonne) for nanocellulose production and frequent clogging of the production system. Various pre-treatments methodologies are evolved to reduce energy consumption and to avoid clogging in homogenizer. One among them, cellulase enzyme pre-treatment, is very popular and highly researched due to eco-friendliness and efficacy. Apart from cellulase enzyme the cellulase secreting fungi as such are being used for ease of handling and to reduce the cost of enzyme processing. Well studied fungi include *Trichoderma* sp. and *Aspergillus* sp. for pre-treatment of cellulosic biomass before homogenization process for production of nanocellulose. Lately, controlled hydrolysis by fungi itself evolved for production of nanocellulose thereby bypassing the homogenization process step. This makes fungi a versatile organism for production of nanocellulose.

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1 Nanocellulose

Cellulose, the most abundant biomass available on Earth, is a biodegradable homo -polymer of β-(1, 4) linked D-glucose units. Cellulose is a straight chain polymer consisting of multiple units of D-glucose linked together in a repeating, overlapping pattern, resulting in a high tensile strength polymer. Cellulose is the main structural component of the primary cell wall of plants, many forms of algae and fungi. For industrial use, cellulose is obtained from wood pulp, agro-biomass and cotton (Satyamurthy and Vigneshwaran 2013). Nanocellulose is a novel biomaterial derived from any cellulosic biomass by various processes viz., mechanical, chemical, biological and in combinations of them. They are very much interesting due to its renewable nature, anisotropic shape, excellent mechanical properties, good biocompatibility, tailorable surface chemistry, and interesting optical properties (Prasad et al. 2015; Abitbol et al. 2016). At least, any one dimension of nanocellulose has to fall in the region of nanometres (1–100 nm). They are classified as nanocrystalline cellulose (NCC) and nanofibrillated cellulose (NFC) according to its aspect ratio. NCC has the aspect ratio less than 100, and in general, called as nanowhiskers due to their elongated whisker shape. In case of NFC, the aspect ratio is more than 100, and in general, they are more than 1000 so that it forms a very long fibrillated structure. Further classifications can be based on the method of preparation, source of the raw material and intended application area. Figure 16.1 shows the overall classification of nanocellulose.

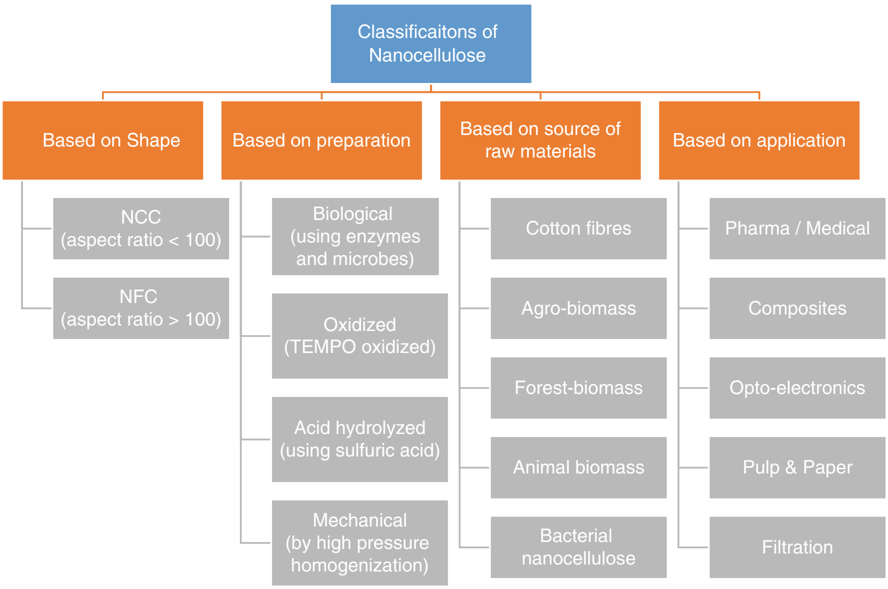

Fig. 16.1 Classifications of nanocellulose

The purest form of nanocellulose could be obtained from cotton fibres and bacterial cellulose, while other raw materials require extensive purification to remove lignin, hemicellulose and other impurities. Nanocellulose can be produced by top-down approach (mechanical/chemical/enzymatic degradation) or bottom-up approach (bacterial cellulose synthesis). The major areas of application of nanocellulose include pulp and paper, polymer film composites, paint and pigments, non-calorific food thickeners and drug delivery system. In spite of established application potential of nanocellulose, the major bottleneck encountered is the requirement of huge amount of energy in production of nanocellulose. An extensive review on this aspect was recently published by our research group (Bharimalla et al. 2015). The NCC and NFC isolated from pure rice straw cellulose via sulfuric acid hydrolysis, mechanical blending and TEMPO-mediated oxidation resulted in 16.9, 12 and 19.7 % yields, respectively. Sulfuric acid hydrolysis produced highly crystalline (up to 90.7 % CrI) rod-like (3.96–6.74 nm wide, 116.6–166 nm long) NCCs with negative surface charges (−67 to −57 mV); Mechanical defibrillated NFCs were 82.5 % crystalline and bimodally distributed in sizes (2.7 nm wide and 100–200 nm long; 8.5 nm wide and micrometers long); and TEMPO mediated oxidation liberated the most uniform, finest (1.7 nm) and micrometer long, but least crystalline (64.4 % CrI) NCCs (Jiang and Hsieh 2013). Figure 16.2 shows the various options of modifying nanocellulose for diversified applications (Dufresne 2013).

2 Cellulose Degrading Fungi

Cellulose is degraded by cellulase enzymes that are highly specific in nature and the product of hydrolysis is glucose. The utility cost of enzymatic hydrolysis is low as compared to acid or alkaline hydrolysis process since enzyme hydrolysis happens at relatively mild conditions viz., pH 4.8 and temperature 45 °C (Sun and Cheng 2002). In general, aerobic fungi and anaerobic bacteria are known to produce cellulase enzyme. In case of aerobic fungi, cellulases are produced as multi component enzyme system comprised usually of three components that act synergistically in the hydrolysis of cellulose; endoglucanases (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and cellobiase (β-glucosidase, EC 3.2.1.91). In case of anaerobic bacteria, large multi protein complexes known as cellulosome are involved in degradation of cellulose and it has about 11 different enzymes aligned on the non-catalytic scaffolding protein that ensure a high local concentration, together with the correct ratio and order of the components. Figure 16.3 shows the action of cellulase enzyme on cellulosic substrate and Fig. 16.4 shows the action of cellulosome on cellulosic substrate (Beckham et al. 2011).

Cellulase has applications in diversified industries including agriculture (for enhanced plant growth and flowering), bioconversion (ethanol from cellulose), detergents (superior cleaning with fibre damage), fermentation (improved aroma of wines), food (clarification of fruit juices), pulp and paper (co-additive in pulp bleaching), and textile (biopolishing of textile fibers) (Kuhad et al. 2011; Salahuddin et al. 2012).

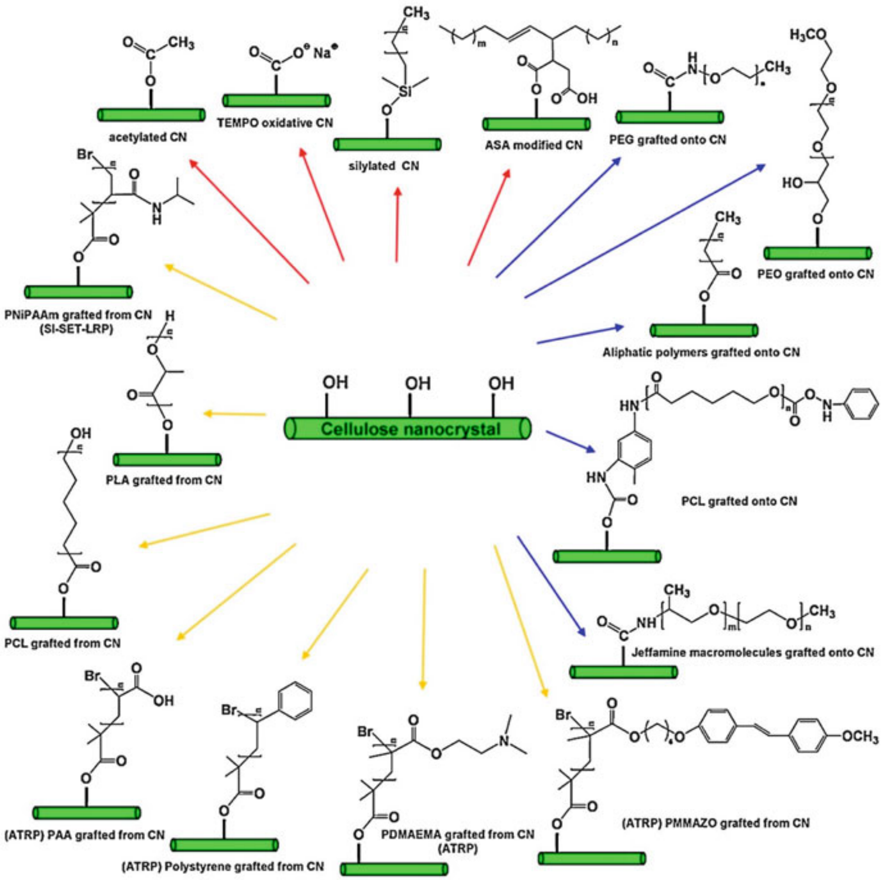

Fig. 16.2 Various modifications of nanocellulose for diversified applications. Reprinted with permission from Dufresne (2013)

3 Production of Nanocellulose by Cellulose Degrading Fungi

The enzymatic hydrolysis of cellulose, particularly hydrogen-bonded and ordered crystalline regions, is a very complex and slow process. Among the two major types of cellulose (algal-bacterial type rich in cellulose Iα crystalline region and cotton–ramie type rich in cellulose Iβ), algal–bacterial type is highly susceptible to cellulase enzyme. The cotton cellulose is recalcitrant due to the dominance of cellulose Iβ structure. In our work (Satyamurthy et al. 2011) we have explored a possibility of controlled hydrolysis of microcrystalline cellulose (MCC) using the fungus *T. reesei* with the yield of 22 %. The penetration of fungus into the ordered regions of MCC during incubation resulted in reduced crystallinity of nanocellulose prepared by microbial hydrolysis compared to that of acid hydrolysis. Figure 16.5 shows the AFM images of nanocellulose prepared by controlled microbial hydrolysis in comparison with that of sulfuric

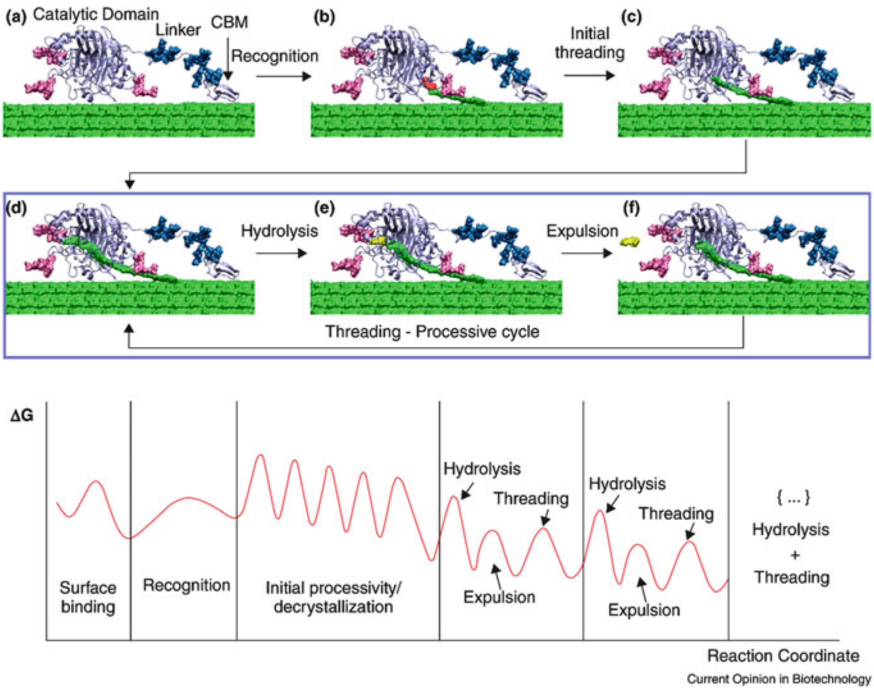

Fig. 16.3 The *Trichoderma reesei* Family 7 cellobiohydrolase (Cel7A) acting on cellulose. Cel7A is comprised of a 36-amino acid CBM, a linker domain with O-glycan (*dark blue*), and a large catalytic domain with N-linked glycan (*pink*) and a 50-Å tunnel for processing cellulose chains (*green*). The cellobiose product is shown in *yellow* (**e**) and (**f**). Here, the putative steps that Cel7A takes to deconstruct biomass and the hypothesized free energy surface for each elementary step (**a**–**f**) is shown. Reprinted with permission from Beckham et al. (2011)

acid hydrolysis process. The soft rot ascomycetes fungus *Trichoderma reesei* is utilized for industrial production of secreted enzymes, especially lignocellulose degrading enzymes. *T. reesei* uses several different enzymes for the degradation of plant cell wall-derived material, including nine characterized cellulases, 15 characterized hemicellulases and at least 42 genes predicted to encode cellulolytic or hemicellulolytic activities (Mari Häkkinen et al. 2014). As this fungus is being exploited for commercial use, nanocellulose production using this fungus also will add a new dimension. Earlier studies reported the successful production of NFC using the endoglucanase enzyme in combination with mechanical shearing and high-pressure homogenization (Henriksson et al. 2007; Pääkkö et al. 2007; Zhu et al. 2011), but, with a lot of energy input.

In our another work, the enriched anaerobic microbial consortium (for cellulase production) is proven to be efficient in hydrolyzing microcrystalline cellulose to produce nanocellulose in a span of 7 days with a maximum yield of 12.3 %. Nanocellulose prepared by this process has a bimodal particle size distribution (43 ± 13 and 119 ± 9 nm) (Satyamurthy and Vigneshwaran 2013). Figure 16.6 shows the AFM image of nanocellulose prepared by anaerobic microbial consortium.

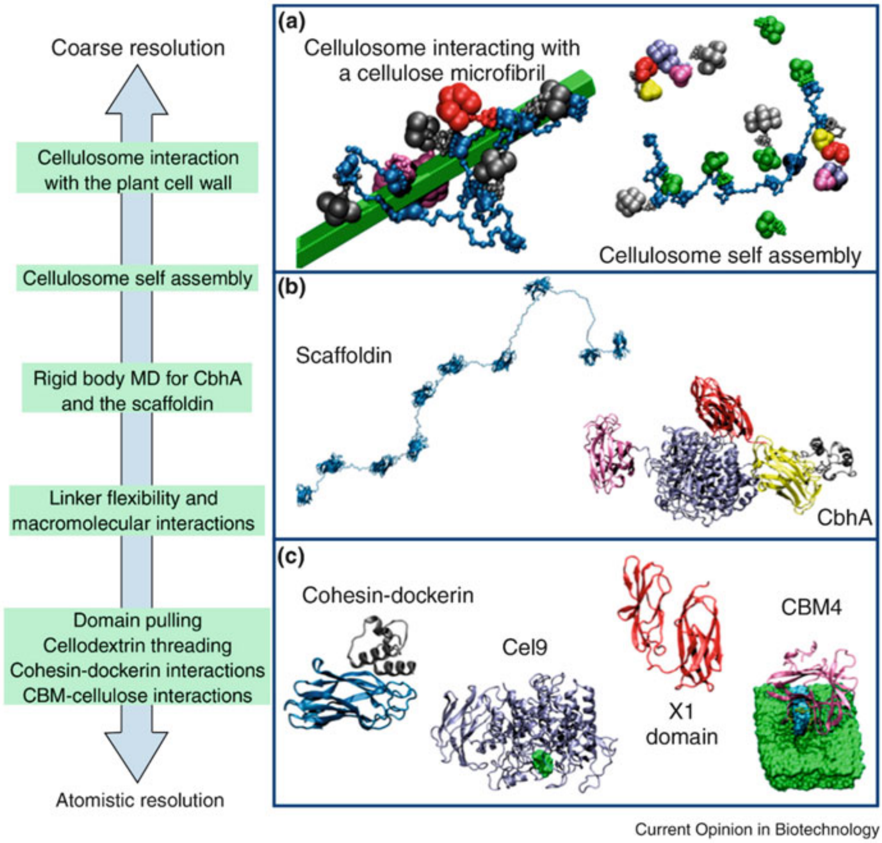

Fig. 16.4 Multi-scale modeling can aid in the understanding of the cellulosomal complex and enzyme–cellulose interactions in the cellulosome. Here are several open questions at various degrees of resolution together with methods to probe each question. (**a**) A simple coarse-grained model has been developed to study self-assembly of the entire cellulosome enzyme complex as a function of enzyme concentration and other relevant variables. (**b**) Rigid body MD enables calculation of solution behavior directly from simulation to compare with SAXS and FRET experiments of the large CbhA enzyme. (**c**) Multiple scientific questions exist at the atomistic scale that can be examined with methods such as rare event simulation to understand mechanisms of threading cellodextrin chains into cellulase tunnels, free energy perturbation methods for relative binding free energies and absolute binding free energies of carbohydrates to cellulases and CBMs, docking calculations to understand the non-covalent binding at the atomic scale, steered MD to understand the work to extend putatively flexible proteins, and REMD to understand intrinsic disorder. Reprinted with permission from Beckham et al. (2011)

4 Purification of Nanocellulose

Purification of nanocellulose becomes a bottleneck while dealing with the microbial process of nanocellulose production. Many of the broth components and fungal secretes are also fall in the nanometer size range that makes it difficult for separation of nanocellulose. The two different options of separation of nanocellulose from the fungal

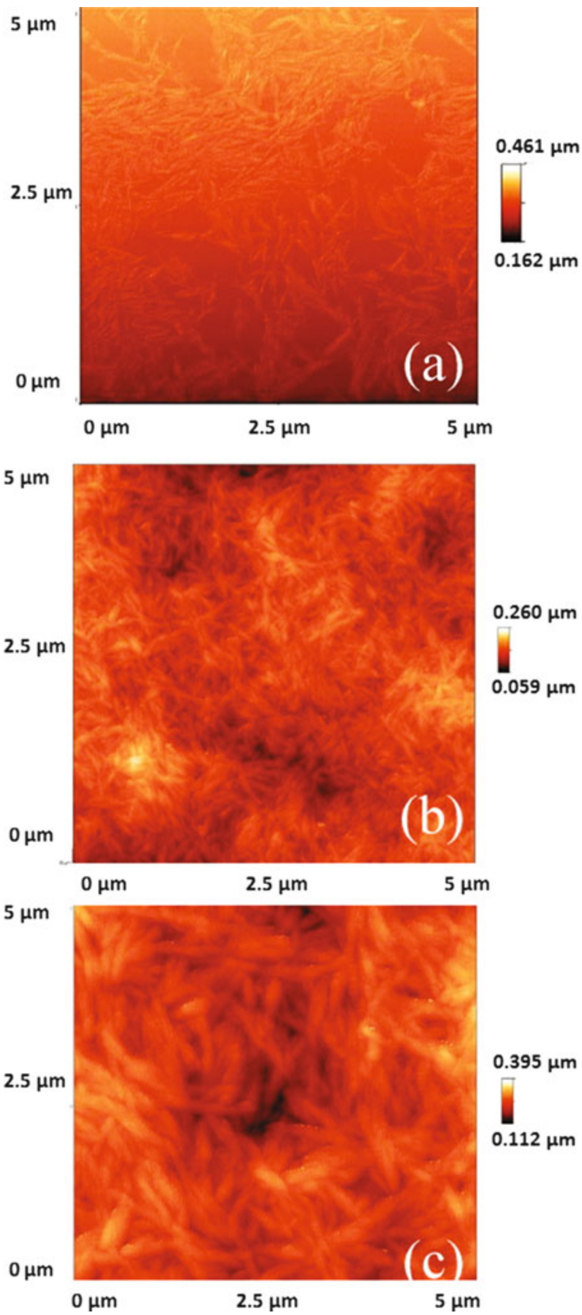

Fig. 16.5 AFM images of the nanocellulose prepared by acid (**a**) and fungal (**b**, **c**) hydrolysis. Reprinted with permission from Satyamurthy et al. (2011)

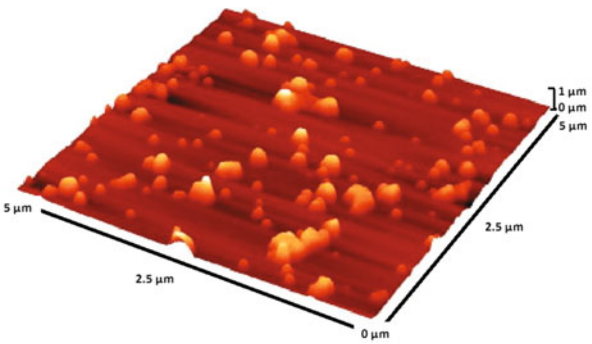

Fig. 16.6 AFM image of spherical nanocellulose prepared using anaerobic microbial consortium. Reprinted with permission from Satyamurthy and Vigneshwaran (2013)

culture and broth components include differential centrifugation and filtration through membrane filters. While differential centrifugation is a tedious and time consuming process, the filtration process suffers due to frequent blocking. Also, since the nanocellulose product size distribution is very wide, the differential centrifugation/filtration techniques could not purify all the nanocellulose that formed during the process. Newer ideas including immobilization of fungal cultures during the controlled hydrolysis process in combination with differential rate of settling of nanocellulose in the broth are being developed as alternate means of purification of nanocellulose. Figure 16.7 shows the overall fermentation system for production of nanocellulose by hydrolysis of cellulose using the fungus *Trichoderma reesei*.

5 Application of Nanocellulose

Three different applications of nanocellulose were recently reviewed (Gómez et al. 2016): (1) nanocellulose as a stabilizing agent, (2) nanocellulose as a functional food ingredient and (3) nanocellulose in food packaging. The last is the most common application of nanocellulose in the food industry. Nanocellulose has potential use as a stabilizing agent in food emulsions, as dietary fiber and to reduce the caloric value of food. Nevertheless, validated standards to characterize the produced nanostructure, quantify its properties and evaluate its toxicity are still required to answer safety and regulatory issues to achieve the incorporation of nanocellulose as a commercial product in the food industry. In another work (Ioelovich 2016), applications of five kinds of nanocellulose, crystalline nanoparticles, amorphous nanoparticles, nanofibrillated cellulose, bacterial nanocellulose, and cellulose nanoyarn that in various areas of care and cure were discussed. The crystalline nanoparticles are applied as multifunctional agents in cosmetic remedies and dentifrices. The amorphous nanoparticles can be used as an antibacterial and hemostatic nanoagent. Nanofibrillated cellulose is characterized by excellent thickening and gel-forming properties. Bacterial nanocellulose finds applications in diverse areas of personal care and biomedicine. Nanoyarn can be used to create new types of wound dressings.

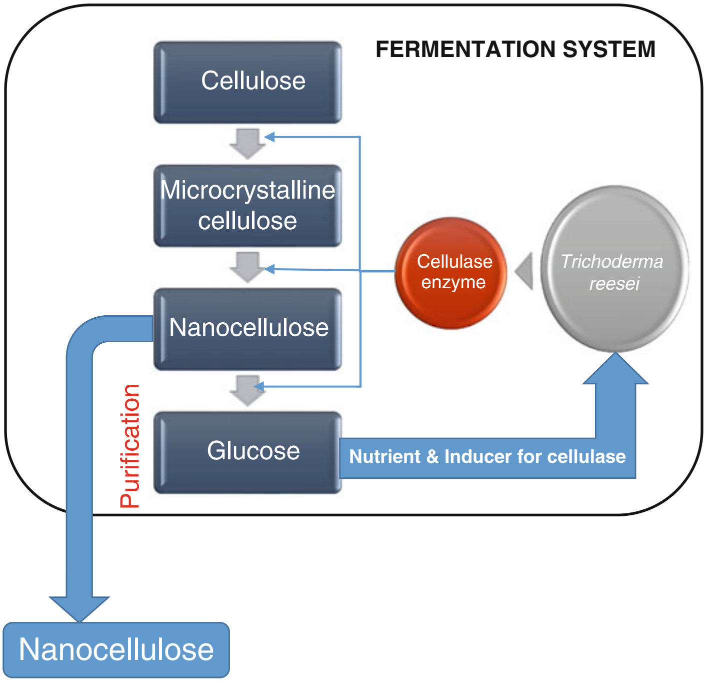

Fig. 16.7 Schematic representation of fermentation system for production of nanocellulose

The other main areas of nanocellulose research including photonics, films and foams, surface modifications, nanocomposites, and medical devices were reviewed in an another work (Abitbol et al. 2016). Nanocellulose, with its ability to form hydrogen bonds resulting in strong network makes it very hard for the molecules to pass through, suggesting excellent barrier properties associated with films made from these material (Nair et al. 2014). In most of the applications dealing with biological system, it is better to have the nanocellulose without any surface modification and without metallic contamination. While the chemically produced nanocellulose inherits the sulfated or carboxylated surface, mechanically produced nanocellulose suffers heavy metal contamination during processing in high energy refining and milling processing. In these circumstances, nanocellulose produced by fungal hydrolysis route offers an excellent alternative as they are bio-compatible and retains the cellulosic nature on its surface.

6 Challenges and Ways Ahead

Table 16.1 shows the comparative aspects of nanocellulose produced by different processes, viz., mechanical, chemical, enzymatic and microbial processes. Each and every process has its own merits and demerits and selection depends on the demand and potential application to be explored.

Table 16.1 Comparison of nanocellulose produced by different processes

Parameters of final product (Nanocellulose)	Methods of preparation			
	Mechanical	Chemical	Enzymatic	Microbial/fungal
Rate of formation	Fast	Fast	Very slow	Slow
Yield (%)	>80	40–60	30–40	10–25
Morphology	NFC	NCC	NCC	NCC
Surface chemistry	Not changed	Sulfated or carboxylated	Not changed	Not changed
Stability	Stable due to fibrillar structure	Highly stable due to very high surface charge	Relatively unstable	Stable due to bound protein and broth components
Ease of operation	Very easy	Difficult	Easy	Easy
Effluent	No effluent generated	Chemical effluent (acidic) generated with high COD	Effluent with high BOD generated	
Cost of production	High	Medium	Very high	High

The cellulose degrading fungi are found to have scope for large scale production of nanocellulose for commercial exploitation. The major challenges are:

(a) To control the size distribution of the product (nanocellulose) in the dynamic production system using cellulose degrading fungi
(b) To increase the yield as increase in substrate concentration act as a limiting factor in a fermentation system
(c) Purification of nanocellulose from the broth substances and the fungal biomass
(d) Efficient control of cellulose degradation process by the cellulase enzyme secreted by fungi.

By overcoming the above said challenges and as governed by the need for eco-friendly system of production, fungal based nanocellulose production could be of the future for diversified bio-based applications.

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