Carbohydrate Polymers 95 (2013) 540–549

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Review

Pullulan: An exopolysaccharide and its various applications

Vipul D. Prajapati*, Girish K. Jani, Simin M. Khanda

Department of Pharmaceutics, SSR College of Pharmacy, Saily-Silvassa Road, Saily, Silvassa, U.T. of Dadra and Nagar Haveli 396230, India

ARTICLE INFO

Article history:
Received 24 December 2012
Received in revised form 3 February 2013
Accepted 21 February 2013
Available online 13 March 2013

Keywords:
Aureobasidium pullulan
Polysaccharide
Biomedical
Diagnostic application
Medical imaging
Linkage

ABSTRACT

Pullulan is a non-ionic polysaccharide obtained from fermentation of black yeast like *Aureobasidium pullulans* and is currently exploited in food and pharmaceutical industries due to its unique characteristics. Due to its properties like non-toxic, non-immunogenic, non-carcinogenic, non-mutagenic, pullulan is being explored for various biomedical applications viz., gene delivery, targeted drug therapy, tissue engineering, wound healing, and also being used in diagnostic applications like, perfusion, receptor, and lymph node target specific imaging and vascular compartment imaging. The unique linkage of α (1 → 4) and α (1 → 6) in pullulan endows this polymer with distinctive physical traits, including adhesive property and the ability to form fibers. This review article presents an historical outline, overview of properties, production, derivatives of pullulan, its versatile applicability and recent advances of pullulan.

Contents

* Corresponding author at: Department of Pharmaceutics and Pharmaceutical Technology, SSR College of Pharmacy, Saily-Silvassa Road, Saily, Silvassa, U.T. of Dadra and Nagar Haveli 396230, India. Tel.: +91 09824284159; fax: +91 0260 2681104.
E-mail address: vippra2000@yahoo.com (V.D. Prajapati).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.02.082

1. Introduction

Biopolymers are the generally water soluble gums, produced by a variety of micro-organism, which have novel and unique physical properties. Because of these properties, these have found a wide range of applications in food, pharmaceutical and other companies. Some of the applications include their use as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubricants, film formers, thickening agents and suspending agents. Advances in technology occur due to the exploitation of properties offered by new polymeric materials like blends, composites, etc. Blend of polymers are prepared by physical mixing of two or more polymers. The resulting blending system has the property superior to any one of the individual polymer (Cheek, 1990; Sun, Wang, & Feng, 1992). Pullulan is one of the polymers obtained from the fermentation medium of the black yeast like *Aureobasidium pullulans* (as it forms a black pigment, melanin so called black yeast) (Bishwambhar, Suneetha, & Ramalingam, 2011). It shows activity against for enzymes such as invertase, amylases, glucose oxidase, β-glusosidase, fructosyltransferase, and small quantities of proteolytic enzyme (Duan, Chi, Wang, & Wang, 2008; Li et al., 2007; Zheng, Campbell, McDougall, & Seviour, 2008). Pullulan was first reported by Bernier in 1958 and the structure was elaborated by Bender et al. in 1959. It comprises of maltotriose units connected by α (1 → 4) glycosidic bond, whereas consecutive maltotriose units are connected to each other by α (1 → 6) glycosidic linkages (Bender, Lechmann, & Wallenfells, 1959).

The application of pullulan is emerging as a source of polymeric materials, which are economical and competitive with the natural gums produced from marine algae and other plants (Suneetha, Sindhuja, & Sanjeev, 2010).

2. Historical outline

Origin of pullulan occurred 6–7 decades earlier i.e. in 1950s. *A. pullulans* was first described as *Dematium pullulans* by De Bary. Bernier was the first to isolate pullulan form *A. pullulans* in 1958. Bender et al. studied the novel polysaccharides in 1959 and named it pullulan. In 1960s, the basic structure of pullulan was resolved (Wallenfels, Bender, Keilich, & Bechtler, 1961). They discovered the enzyme pullulanase, which hydrolyses α-(1 → 6) linkages in pullulan and converts to maltotriose. Thus, pullulan is viewed as α-(1 → 6) linked polymer of maltotriose subunits (Fig. 1).

Pullulan has the safe history of use in Japan as a food ingredient and as pharmaceutical bulking agent. The main use of pullulan has been as a glazing agent having oxygen barrier properties (Carolan, Catley, & McDougal, 1983). It has generally regarded as safe (GRAS) status in US for a wide range of applications. Human volunteer studies have only reported the abdominal fullness at doses of 10 g pullulan per day with some mild gastrointestinal symptoms at higher doses. Pullulan is accepted for use as an excipient in pharmaceutical tablets and is listed in the Japanese Standards for Ingredients for drugs (Mishra, Vuppu, & Rath, 2011; Rekha & Sharma, 2009).

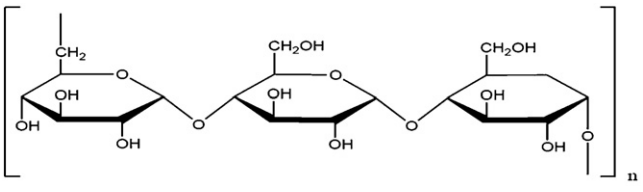

Fig. 1. Chemical structure of pullulan.

The commercial production of pullulan began in 1976 by the Hayashibara Company, located at Okayama, Japan. Pullulan production was an outgrowth of starch syrup production, noted in 1883. Pullulaln films were commercialized by Hayashibara in 1982.

3. Properties of pullulan

Dry pullulan is white to off-white tasteless, odorless powder which forms a viscous non-hygroscopic solution when dissolved in water at 5–10%. Pullulan starts to decompose at 250 °C and chars at 280 °C. It is highly soluble in water, dilute alkali, insoluble in alcohol and other organic solvents expect dimethylsulfoxide and formamide. As pullulan is highly water soluble so it can be used as a carrier for drug and it helps in controlled release of drug in plasma. It has the molecular weight within the range of 5000–9,000,000 g/mol with straight unbranched chain and is very flexible molecule having the property of "random–coil" (depending on sedimentation coefficient and intrinsic viscosity measurement). Pullulan is non-toxic, non-mutagenic, non-carcinogenic, odorless, tasteless, and edible (Kimoto, Shibuya, & Shiobara, 1997).

Pullulan has a considerable mechanical strength and other functional properties like adhesiveness, film formability, enzymatically mediated degradability (Shingel, 2004).

Pullulan is biodegradable, impermeable to oxygen, and is not attacked by the digestive enzymes of the human gut, hence can be used as carrier for oral delivery of drug. Pullulans solutions have relatively low viscosity, resembling gum Arabic (Tsijisaka & Mitsushashi, 1993). It can be used as low-viscosity filler in beverages and sauces. The viscosity of pullulan solution does not change with heat, change in pH, and most metal ions including sodium chloride.

4. Production of pullulan

Pullulan is usually produced on industrial scale by the fermentation of liquefied starch under specified parameters using a specific, not genetically modified, non-pathogenic and non-toxigenic strain of *A. pullulans*.

4.1. Pullulan production by fermentation

The production of pullulan depends on the fermentation parameters viz., the morphological state and the fungal strains. In commercial production (Tsijisaka & Mitsushashi, 1993), is cultivated on medium, comprises of starch hydrolysates of dextran

equivalent to 40–50, at 10–15% concentration. The fermentation medium consists of peptone, phosphate and basal salts. The pH of the culture media is initially adjusted to pH 6.5, which falls during the first 24 h to a pH of 3.5. Maximal growth of culture media occurs within 75 h. Optimal pullulan yields are obtained within about 100 h. Cultures are stirred, aerated, and the temperature is maintained at temperature of 30 °C. Yield of greater than 70% of initial substrate is claimed. Culture conditions and strain selection are important parameters in obtaining high molecular weight pullulan, which is relatively free of melanin. *A. pullulans* cells are removed from media by filtration of diluted culture broth. Melanin is removed by treating it with activated charcoal. Pullulan is recovered and purified by precipitation with organic solvents, particularly alcohol. It may be further purified by the use of ultrafiltration and ion exchange resins.

Youssef et al. reported the production of pullulan by using various strains of *A. pullulans* using sucrose and glucose in shaking flask and stirred tank fermenters. They reported maximum concentration of pullulan at 31.3 g/L with pullulan productivity of 4.5 g/L per day (Youssef, Biliaderis, & Roukas, 1998). In another study Shabtai and his co-workers produced pullulan in a two stage fermentation process with increased productivity. In the first step, fermentation was carried out using soyabean oil as a carbon source and glutamate as nitrogen source, at pH of 4.5, which resulted in the concentration of pullulan at 15 g/L. In the second stage, the cells were shifted to production, which was carried out using sucrose as a carbon source with nitrogen limitation. They reported the concentration of pullulan about 35 g/L in 50 h (Thirumavalavan, Manikkadan, & Dhanasekar, 2009). Recently, Roukas with his co-workers observed maximum concentration of pullulan of 30 g/L in an air-lift fermenter at an aeration rate of 2 vvm (vol/vol/min). West and Strohfus (2001) produced the pullulan in two cycle process of 165 h, using either agar or calcium alginate for immobilized cell system. The cells which were immobilized in alginate gave a higher production of pullulan with 4.2 mg pullulan per gram cells per hour during the first cycle and 4.6 mg per gram cells per hour during the next cycle (Goksungur, Ucan, & Guvenc, 2004).

4.1.1. By using coconut by-products

Thirumavalavan et al. synthesized pullulan from coconut by-products using *A. pullulans*. The strain was maintained on agar slants at 4 °C and subcultured every fortnight time interval. The seed medium comprises of sucrose, potassium dihydrogen phosphate, yeast extract, ammonium sulfate, sodium chloride and distilled water. The medium was autoclaved for 15 min at 121 °C, cooled and the pH was adjusted to 7. Then the culture was incubated at 30 °C for 36 h in a rotary shaker incubator at 200 rpm. The highest concentration of pullulan was 54 g/L in coconut milk (Israilides, Smith, & Bambalov, 1993).

4.1.2. By beet molasses

Goksungur and his co-workers produced pullulan by beet molasses using *A. pullulan*. The media comprised of sucrose, ammonium sulfate, yeast, potassium dihydrogen phosphate, magnesium sulfate, and sodium chloride. They reported the highest pullulan concentration 35 g/L obtained in molasses treated with sulfuric acid and activated carbon (Shin, Kim, Lee, Cho, & Byum, 1989).

4.1.3. From agro-industrial waste

Pullulan can be sysnthesized from a various carbohydrate substrates incorporated into defined (synthetic) or non-defined media. The latter covers the agro-industrial wastes, which have been shown to be suitable for pullulan production (Israilides et al., 1998; Israillides, Scanlon, Smith, Jumel, & Harding, 1994). The utilization of these substrates for the production of pullulan seems to be economically advantageous and economically sound.

Different fermentation parameters for the production of pullulan have been studied with defined substrates viz. glucose and sucrose, but the results from the agro-industrial wastes have shown that higher or similar yield of pullulan can be obtained as compared to conventional substrate (Saha & Bothast, 1993). Also, pullulan produced by such fermentations is characterized by heterogenecity of both composition and molecular weight (Israilides, Vlyssides, Mouraferi, & Karvouni, 1977). Following are the agro-industrial wastes which have been used for the production of pullulans:

- Grape skin pulp (Israilides et al., 1977).
- Molasses (Israilides et al., 1977).
- Starch waste (Shabtai & Mukmenev, 1995)
- Olive oil wastes (Israilides et al., 1977; Roukas & Biliaderis, 1995).
- Carob pod (Roukas & Biliaderis, 1995).

4.2. Biosynthesis of pullulan

4.2.1. Exopolysaccharide produced by A. pullulans

Even in the first work on pullulan biosynthesis, researchers observed that the culture produced two different exopolysaccharides. One of these polymers corresponds to pullulan, and the second is frequently described as a water-insoluble jelly like material. An electron microscopy study revealed that both pullulan and the insoluble polysaccharide are localized on an outer surface of the chlamydospores, the cells that were considered as the main polysaccharide producer on non growth media. The highly dense peripheral layer was ascribed to the chain of pullulan arranged in a network covering the inner layer of β-(1 → 3)-glucan composed of glucose and mannose (Simon, Caye-Vaugien, & Bouchonneau, 1993). The mechanism of biosynthesis of these jelly like glucans is associated with the pullulan e1laboration, though there were indications that the elaboration of insoluble exopolysaccharide is dependent on genetic type of *A. pullulans*. In particular, it is not clear yet whether environmental conditions, for example, pH or morphological changes of the cells are responsible for its extracellular elaboration (Imshenetskii et al., 1983).

4.2.2. Influence of pH and cell morphology

Relatively low initial pH (pH 2.5) suppresses synthesis of pullulan but stimulates elaboration of the insoluble glucan. An optimal value of pH for the pullulan production lies in the range between 5.5 and 7.5 (Lee et al., 2001). There were only a few reports where highest pullulan content was achieved by cultivating the microorganism in acidic pH. It is of interest to note that the optimal pH established for the biomass growth is 4.5 or lower. This difference in optimal values of pH for the pullulan synthesis and cell mass growth indirectly correlates with the independent character of these two processes (Kondratyeva, 1981). However, the relationship between morphology and the polysaccharide-producing capacity of the culture cannot be ignored since the polysaccharide elaboration is known to be associated with the specific cell morphology (Simon et al., 1993), though the exact ce1llular type responsible for pullulan synthesis is still a matter of debate. In an overwhelming number of studies, pullulan elaboration was found to occur only with the yeast-like morphology of *A. pullulans* (Campbell, Siddique, McDougall, & Seviour, 2004), whilst in other several papers the ability to synthesize the polysaccharide was the characteristic of the chlamydospore population. At least there is convincing agreement among researchers that pH provokes morphological changes of cells, which in turn may additionally differentiate biosynthesis routes. The yeast-like cells at neutral pH produce pullulan of a very high molecular weight (Lee et al., 2001), whilst combined cultivation of the *mycelial* and the yeast-like cellular forms can be beneficial for high pullulan concentration (Roukas & Biliaderis, 1995).

4.2.3. Mechanism of pullulan biosynthesis

Although many investigations on biochemical mechanisms of exopolysaccharide biosynthesis in bacteria have been carried out (Degeest & Vuyst, 2000), relatively little is understood about the mechanisms of pullulan biosynthesis in *A. pullulans*. If the pullulan biosynthesis and regulation in *A. pullulans* are elucidated, it will be very easy to enhance pullulan yield using molecular methods. Pullulan can be synthesized from sucrose by cell-free enzymes of *A. pullulans* when both adenosine triphosphate (ATP) and uridine diphosphate (UDP)-glucose are added to a reaction mixture (Shingel, 2004). Chi et al. reported that the size of UDP-glucose pool and glucosyltransferase activity in the cell of *A. pullulans* Y68 obtained in their laboratory may be correlated with high pullulan production (Chi et al., 2009). Therefore, effects of different sugars on pullulan production, UDP-glucose (UDPG) pool, and activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase, and glucosyltransferase in the cells of *A. pullulans* Y68 were investigated (Duan et al., 2008). It was found that more pullulan is produced wh1en the yeast strain is grown in the medium containing glucose than when it is cultivated in the medium supplementing other sugars. However, Chi et al. concluded that when more pullulan is synthesized, less UDP-glucose is left in the cells of *A. pullulans* Y68. High pullulan yield is positively related to high activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase, and glucosyltransferase in *A. pullulans* Y68 grown on different sugars (Chi et al., 2009). A pathway of pullulan biosynthesis in *A. pullulans* Y68 was proposed based on different studies (Chi et al., 2009). It is thought that the lower amount of pullulan produced by *A. pullulans* Y68 from fructose and xylose may be caused by the longer biosynthetic pathway leading from fructose and xylose to UDP-glucose. It is thought that most of UDP-glucose is used to synthesize pullulan when the glucosyltransferase activity is very high, leading to very low UDP-glucose level in the yeast cells. This may imply that very high glucosyltransferase activity is the unique characteristic of *A. pullulans* Y68 which can produce high yield of pullulan. Because the phosphoglucomutase and UDPG-pyrophosphotylase activity in the yeast cells grown in the medium containing glucose is also very high, UDP-glucose is synthesized continuously to supply the precursors for high pullulan synthesis when the very high glucosyltransferase activity occurs in the cells of *A. pullulans* Y68. However, high level of UDP-glucose is left when the yeast cells are grown in the medium containing xylose and fructose, respectively, due to low glucosyltransferase activity. Therefore, it is believed that the proposed pathway of pullulan biosynthesis will be helpful for the metabolism.

5. Semi synthetic derivatives of pullulan

Pullulan can be easily derivatized in order to enhance its activity and widen the window of its applications. Pullulan can be derivatized in various ways such as;

5.1. Chemical modification

Pullulan can be derivatized to enhance its applications by grafting different chemical structures on the backbone. Pullulan consists of nine hydroxyl groups for the substitution reactions on the repeating unit. Pullulan's utility can be improved using grafting of different chemical groups on its hydroxyl groups as they cab be easily substituted. The relativities of these hydroxyl groups also depend on the polarity of the solvent and the reagents. The hydroxyl groups of pullulan were subjected to various chemical reactions, leading to the formation of a large number of derivatives, which are given in Table 1 (Bataille, Meddahi-Pelle, Visage, Letourneur, & Chaubet, 2011, chap. 4).

Chemical modification includes:

Table 1
Schematic chemical structure of the most common pullulan derivatives.

Type of reaction	Schematic chemical structure of substituted pullulan (P-OH)
Etherification	P-O—CH_3 (permethylation)
	P-O—$(CH_2)_{2\text{-}3}$—CH_3 (alkylation)
	P-O—CH_2—COOH (carboxymethylation)
	P-O—$(CH_2)_{2\text{-}3}$—CH_2—NH_3^+ (cationization)
Etherification	P-O—CH_2—CH_2—CN (cyanoethylation)
	P-O—(CH_2)1-4-Cl (chloroalkylation)
	P-O—CH_2—CH_2—(S=O)—CH_3 (sulfinyl)
	P-O—CH_2—CH_2—CH_2—SO_3Na
	P-O—CH_2—CH_2—N(CH_2CH_3)
Esterification	P-O—CO—CH_2—CH_2—COOH (succinoylation)
	PA-O—CO—CH_2—CH_2—CO-sulfodimethoxine
	P-O—CO—CH_2—CH_2—CO-cholesterol
	P-abietate
	P-stearate
	PA-folate
	P-cinnamate
	P-biotin
Urethane derivatives	P-O—CO—NH—CH_2—CH(OH)—CH_3
	P-O—CO—NH—CH_2—CH_2—NH_3^+
	P-O—CO—NH—R (R = phenyl or hexyl)
	P-O—CO—NH-phenyl
Chlorination	P-CH_2—Cl (C_6 substitution)
Sulfation	P-O—SO_3Na
Azido-pullulan	P-CH_2—N_2
Oxidation	P-COOH (C_6 oxidation)
	Glycosidic ring opening (periodate oxidation)
CMP/hydrazone derivative	P-O—CH_2—CO—NH-doxorubicin
	P-O—CH_2—CO—NH-antibody

5.1.1. Carboxymethyl pullulan

Carboxymethylation is the most widely used reaction done on the neutral polysaccharides in order to allow further chemical modification or to favor the solubility in the aqueous solutions. The hydroxyl groups of the pullulan are activated as alcoholate in the alkaline aqueous solution to allow the nucleophilic substitution of chloride from monochloroacetic acid. Several works has been done to understand the behavior of self-assembled or cross-liked carboxymethyl pullulan (Bataille, Huguet, Muller, Mocanu, & Caprov, 1997; Henni-Silhadi et al., 2008; Legros, Dulong, Picton, & Cerf, 2008; Souguir, Roudesli, Picton, Cerf, & About-Jaudet, 2007).

5.1.2. Sulfation

With the aim of developing a new alternative to heparin, pullulan was derivatized by reacting with sulfur. The final property of sulfated pullulan depends on the temperature, solvent and duration of action and the reagent used for the sulfation. Mahner et al. reported a homogenous distribution of the sulfate along both polysaccharidic backbones (Mahner, Lechner, & Nordmeier, 2001). Alban et al. confirmed these results by determining the degree of the sulfation of the hydroxyl group occurred in the order C6 > C2 > C3 > C4 irrespective of the weight of the pullulan (Alban, Schauerte, & Franz, 2002). The sulfated pullulans were obtained by stepwise sulfation of pullulans with SO_3–pyridine complex in dimethyl formamide (DMF) at 75 °C and 95 °C for 3–8 h, in which all the pullulans having the molecular weight 50 kDa (soluble in DMF) and 200 kDa (insoluble in DMF) were used.

6. Application of pullulan

6.1. Biomedical and pharmaceutical application

6.1.1. Tissue engineering and grafting

The bulk and surface properties of bio materials used for medical implants have been shown directly influences and some cases control the dynamic interaction that takes place at tissue implant interface. These characteristics and changes in characteristics that

may takes place over time in vivo should be known for designing biomaterial for specific applications and the same thing can be easily done for the pullulan. Carboxymethylated pullulan conjugated with heparin was developed by various groups and its properties toward tissue engineering applications were investigated. Covalent conjugation of pullulan with an interferon–water-soluble low-molecular-weight recombinant protein that possesses both antiviral and immunoregulatory activity allows one to preserve the biological activity of the drug while enhancing its liver accumulation (Na et al., 2006). Surface modification is a major tool for the tissue engineering purposes. Nine hydroxyl groups are available for substitution reactions on the repeating unit. These nine hydroxy groups can be easily substituted by grafting different chemical groups, which increases the weight of pullulans due to grafting of concerned chemical groups on it. Grafting ratio and efficiency can calculated using formula 1, 2 and 3 (Gao, Yu, Wang, Chang, & Tian, 1998). They are distinguished by their position on the glucosidic moiety OH-2, OH-3, OH-4 and OH-6 with the ratios 3, 3, 1 and 2 respectively and their substitution is listed in Table 1. Heparin-conjugated pullulan inhibited the proliferation of smooth muscle cells in vitro and thus can be used for the proliferation of vascular endothelial cells and to inhibit the proliferation of smooth muscle cells.

$$\text{Grafting ratio}\,(\%G) = \frac{\text{weight of the grafted chain}}{\text{weight of pullulan}} \times 100 \quad (1)$$

$$\text{Grafting efficiency}\,(\%E) = \frac{\text{weight of the grafted chain}}{\text{weight of polymer formed}} \times 100 \quad (2)$$

$$\text{Grafting ratio}\,(\%G) = \frac{\text{weight of the polymer used}}{\text{weight of the monomer used}} \times 100 \quad (3)$$

6.1.2. Pullulan as a carrier for drug delivery

To achieve drug delivery, stimuli-sensitive polymer systems have been intensively exploited as candidate materials (Kin, Cho, & Yuk, 2001; Kurkuri & Aminabhavi, 2004). Most part of pH and temperature sensitive microspheres used for the controlled delivery of drugs are not biodegradable. As studied by Gheorghe et al. in order to confer their temperature sensitivity, poly (Nisopropylacrylamide-co-acrylamide) was grafted onto pullulan microspheres. Then, the pH-sensitive units (—COOH) were introduced by reaction between the remaining —OH groups of the pullulan with succinic anhydride. The grafted pullulan microspheres are more hydrophilic than pullulan microspheres, their swelling degree as well as water regain increase significantly (Gheorghe, Marieta, & Paolo, 2008). Thus a pH and temperature sensitive pullulan microspheres for controlled release of drugs can be prepared (Gheorghe et al., 2008). The use of pullulan, a member of the extracellular polysaccharide family often used in the last decade in pharmaceutics confers to microspheres stability, biocompatibility, and biodegradability (Fundueanu et al., 2003; Mocanu, Mihai, Le Clerf, Picton, & Muller, 2004).

6.1.2.1. Liver targeting of drug loaded pullulan. Liver targeting study focuses on the blood compatibility of the cationic pullulan, physico-chemical characterization, and uptake of nanocomplex by hepatocytes and in vitro transfection. Liver targeting can be achieved by using drug loaded pullulan. Xi and his co-workers studied the liver binding affinity of the modified pullulan in vitro in hepatocytes and in vivo in mice (Xi et al., 1996). The purpose of the study was to actively target interferon (IFN) to the liver through its chemical conjugation with pullulan, a water-soluble polysaccharide with a high affinity for the liver. Chemical conjugation of IFN with pullulan was achieved by a cyanuric chloride method. Following intravenous injection of the conjugates to mice, their body distribution and the activity of an IFN-induced enzyme, 2′,5′-oligoadenylate (2-5A) synthetase in the liver and other organs, were evaluated. The results obtained showed that the cyanuric chloride method enabled to prepare an IFN–pullulan conjugate that retained approximately 7–9% of the biological activity of IFN. Pullulan conjugation enhanced the liver accumulation of IFN and the retention period with the results being reproducible. When injected intravenously to mice, the IFN–pullulan conjugate enhanced the activity of 2-5A synthetase in the liver. The activity could be induced at IFN much lower than those of free IFN injection. In addition, the liver 2-5A synthetase induced by conjugate injection was retained for 3 days, whereas it was lost within the first day for the free IFN-injected mice. The final conclusion made after the study was that IFN–pullulan conjugation was promising for IFN targeting to the liver with efficient exertion of its antiviral activity therein (Xi et al., 1996).

6.1.2.2. Pullulan based anti-cancer drug. Pullulan can be used for tumor cell targeting. Scomparin et al. designed two new anticancer polymers for tumor cell targeting. Two new anticancer polymer therapeutics were designed for tumor cell targeting. The bioconjugates were synthesized by pullulan derivatization with either doxorubicin or doxorubicin and folic acid. Pullulan was activated by periodate oxidation and functionalized by reductive conjugation with cysteamine and 1.9 kDa PEG $(NH_2)_2$. The cysteamine thiol groups were conjugated to doxorubicin through a pH-sensitive hydrazone spacer while the pending PEG-NH_2 functions of one derivatized pullulan batch were conjugated to folic acid to obtain one of the two polymer therapeutics. The reaction intermediates and the final products were characterized by mass spectrometry, UV–vis analysis and reverse phase and gel permeation chromatography. The folic acid-free derivative [(NH_2 PEG)-Pull-(Cyst-Dox)] contained 6.3% (w/w) doxorubicin while the folic acid-doxorubicin-coupled derivative [(FA-PEG)-Pull-(Cyst-Dox)] contained 6% (w/w) doxorubicin and 4.3% (w/w) folic acid. Photon correlation spectroscopy showed that (NH_2 PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) assembled into particles of about 150 and 100 nm diameter, respectively. The two bioconjugates displayed similar drug release profiles either at pH 7.4 buffer or in plasma, where less than 20% of doxorubicin was released within three days. At pH 5.5, both conjugates underwent complete drug release in about 40 h. In vitro studies carried out with KB tumor cells over-expressing folic acid receptor showed that both free doxorubicin and (FA-PEG)-Pull-(Cyst-Dox) were rapidly taken up by the cells, while the internalization of the non-folated derivative was significantly slower. Cell viability studies did not show relevant difference between the two bioconjugates. After 72 h of incubation with folic acid receptor non-expressing MCF7 cells, the IC_{50} values of doxorubicin (NH_2PEG)-Pull-(Cyst-Dox) and (FA-PEG)-Pull-(Cyst-Dox) were 0.3 μM, 1.2 μM and 3.1 μM, respectively. After incubation with KB cells over-expressing folic acid receptor, the IC_{50} values were 0.4 μM, 1.8 μM and 1.1 μM, respectively. Pharmacokinetic studies showed that 4 h after intravenous administration of the conjugates to Balb/c mice about 40% of the administered drug equivalent dose was present in the bloodstream while in the case of unconjugated doxorubicin, 80% of the drug was cleared within 30 min.

These findings suggest that the novel doxorubicin–pullulan bioconjugates possess suitable properties for passive tumor targeting. On the other hand, folic acid conjugation has been found to have limited effect on selective cell up-take (Scomparin, Salmosa, Bersani, Satchi-Fainaro, & Caliceti, 2011).

6.1.2.3. Pullulan as a carrier for gene delivery. Gene therapy is another area where the application of pullulan is being explored. Gene delivery is usually mediated by endocytic pathway. Efforts for gene therapy using virus have been performed but the major drawback that viruses are known to be immunogenic, disease

causing and can be hazardous. So attempts to develop non-viral vectors are taken and cationic derivatives of natural polymers are investigated. Pullulan being biocompatible and non-toxic is investigated for gene delivery application. Pullulan derivative which has metal chelating residues and mixed with a plasmid DNA in aqueous solution containing Zn^{2+} ions to obtain the conjugate of pullulan derivative and plasmid DNA with Zn^{2+} coordination. Liver targeting of plasmid DNA was achieved through conjugation of pullulan derivatives with chelate residues based on metal coordination. Triethylenetetramine (Ti), diethylenetriamine pentaacetic acid (DTPA), and spermine (Sm) was chemically introduced to pullulan, a polysaccharide with an inherent affinity for the liver, to obtain various pullulan-Ti, pullulan-DTPA, and pullulan-Sm derivatives. Irrespective of the type of pullulan derivatives, intravenous injection of the pullulan derivatives–plasmid DNA conjugates with Zn^{2+} coordination significantly enhanced the level of gene expression only in the liver to a significant greater extent than that of free plasmid DNA. The enhanced gene expression by the pullulan-DTPA–plasmid DNA conjugate was specific to the liver and the level was significantly higher than that of the pullulan-DTPA–plasmid DNA mixture. Thel1evel of gene expression depended on the percentage of chelate residue introduced, the mixing ratio of the plasmid DNA–DTPA residue in conjugate preparation, and the plasmid DNA dose. The gene expression induced by the conjugate lasted over 12 days after injection. A fluorescent-microscopic study revealed that the plasmid DNA was localized at the liver after injection of the pullulan-DTPA–plasmid DNA conjugate with Zn^{2+} coordination. Pre-injection of both arabinogalactan and galactosylated albumin suppressed significantly the liver level of gene expression, in contrast to that of mannosylated albumin, indicating that the plasmid DNA in the conjugate was transfected at hepatocytes. It was concluded after the study that the Zn^{2+}-coordinated pullulan conjugation is a promising way to enable the plasmid DNA to target to the liver for gene expression as well as to prolong the time duration of gene expression (Hosseinkhani, Aoyama, Ogawa, & Tabata, 2002).

6.1.3. Medical imaging

Recently nanotechnology has been used for earlier detection of cancerous cell in the body. Quantum dots, which are the nano-size semiconductor particles has attracted many scientists in biological field. They are used as fluorescent probes for cell tracking. Hasegawa et al. developed cholesterol pullulan and amino group modified cholesterol pullulan nanogels for the delivery of quantum dots into cells in comparison to conventional cationic liposome which has the disadvantage of forming aggregates ones gets into the cells. Nanoparticles were prepared by mixing nanogels of derivatized pullulan with quantum dots. They reported the intensity of fluorescence per cell and compared with liposomal-quantum dots complex. The particles with higher number of amino group showed fluorescence up to 3–4 times more than that of control. They concluded that cellular uptake of cholesterol pullulan was enhanced by introducing cationic groups and simultaneously the quantum dot's better than the conventional cationic liposomes and these nanoparticles could be a fluorescent probe for medical imaging (Nobuyuki, Takao, Michiko, Yasuhiko, & Kazunari, 2005).

6.2. Plasma expander

Pullulan was also explored as a potential blood-plasma substitute like that of dextrans. Polymers which are highly water soluble in nature can be used as plasma expanders and pullulan is water soluble polymer. It has been reported that pullulan to be used as plasma expander with molecular weight of about 60 kDa. It was observed that pullulan having high molecular weight increased the venous pressure whereas low molecular weight pullulans were rapidly excluded from the organism leaving the stage of secondary hemorrhagic shock. Thus, pullulan to be used as plasma expander should have an effective therapeutic range of molecular weight. Shingel and his co-worker developed an anionically modified pullulan via gamma irradiation which was used as a base for blood-plasma substitute. γ-Ray-irradiated pullulan macromolecules acquire properties of an anionic polyelectrolyte and, upon aggregation with the oppositely charged surfactant cetyltrimethylammonium hydroxide, are found to precipitate according to their molecular weight. This provides a convenient means for obtaining polymer fractions with a narrower molecular-weight distribution than those of the original samples. The method can be employed to obtain fractions of radiation modified pullulan required in the production of a blood-plasma substitute. Anionic properties of γ-ray irradiated pullulan also manifest themselves in interactions with sodium dodecyl sulfate (SDS) in aqueous solution, which result in a significant change in the viscous behavior of the polysaccharide. Upon an increase in the concentration of γ-ray-irradiated pullulan in an SDS solution, the reduced viscosity of the polymer first increases and, upon reaching a certain concentration, C^*, decreases. The C^* values were found to be dependent on the molecular weight of the polymer. The phenomena observed are discussed in terms of the general theory of polymer solutions within which C^* is treated as a critical concentration at which interpenetration of polymer molecules becomes important. Unperturbed dimensions of γ-ray-irradiated pullulan macromolecules were estimated on the basis of experimental viscosimetric data (Shingel & Petrov, 2002).

6.3. Molecular chaperons

Moleculaes having the chaperon like activity are able to catch and release proteins. Molecular chaperons bind to denatured proteins in order to prevent irreversible aggregation. Then chaperon molecules release the proteins. Water soluble polymers such as polyethylene oxide (PEO) have been tried to increase the recovery yield of parent protein during refolding (Cleland, Hedgepeth, & Wang, 1992). These polymers prevent the aggregation of proteins by blocking their hydrophobic surface. Nomura et al. developed hydrophobized pullulan nanogels having the properties of molecular chaperons. They reported the release of complexed proteins from the nanogels in their refolded forms in the presence of cyclodextrins. They concluded that these amphiphilic nanogels trap the denatured proteins and cyclodextrin acts as an effector molecule to control the binding ability of chaperon molecule to proteins. Molecular chaperone-like activity for protein refolding was investigated using nanogels of self-assembly of cholesterol-bearing pullulan. Nanogels effectively prevented protein aggregation (i.e. carbonic anhydrase and citrate synthase) during protein refolding from GdmCl denaturation. Enzyme activity recovered in high yields upon dissociation of the gel structure in which the proteins were trapped, by the addition of cyclodextrins. The nanogels assisted protein refolding in a manner similar to the mechanism of molecular chaperones, namely by catching and releasing proteins. The nanogels acted as a host for the trapping of refolded intermediate proteins. Cyclodextrin is an effector molecule that controls the binding ability of these host nanogels to proteins. The present nanogel system was also effective at the renaturation of inclusion body of a recombinant protein of the serine protease family (Nomura et al., 2003).

6.4. Hydrophobized pullulan conjugates for drug delivery

Pullulan hydrogels as drug delivery systems in the form of microgels and nanogels was studied. Slow release of drug into

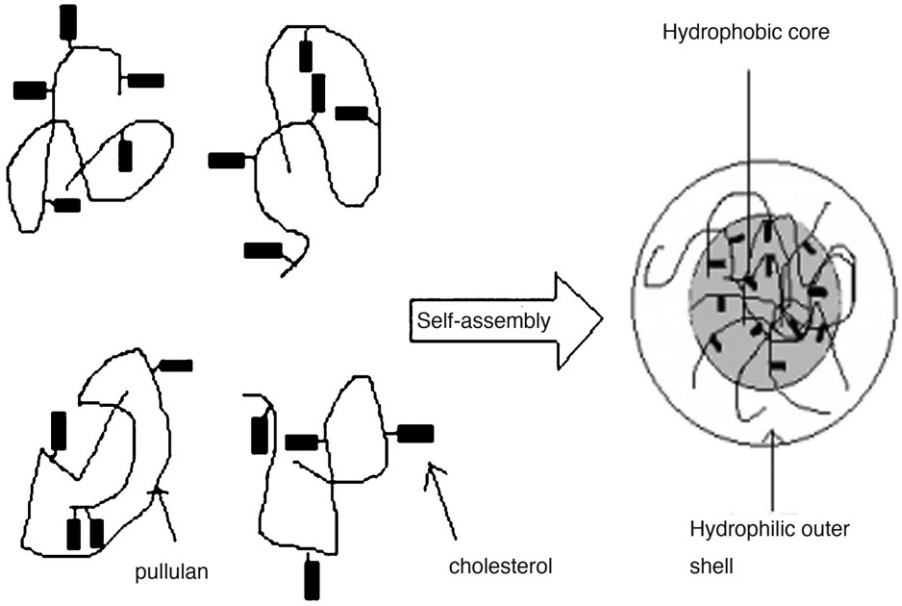

Fig. 2. Formation of cholesterol-pullulan conjugate-based nanoparticles by self-aggregation in aqueous solution.

the plasma helps in attaining the therapeutic benefits (Wooram, Kyoung, Byoung-chan, Young-Heui, & Kun, 2010). In order to develop a novel self-assembly as a means of cancer cell targeting, self-organized nanogels were prepared by them from acetylated hyaluronic acid with low molecular weight (AC-HA_{LM}). Three samples were obtained (AC-HA_{LM}1, 2 and 3) with degrees of acetylation, 0.8, 2.1, or 2.6 acetyl groups per unit (2 glucose rings) of HA_{LM} to control their hydrophobicity. The mean diameters of AC-HA_{LM}2 and 3 were less than 300 nm with unimodal size distribution, while that of AC-HA_{LM}1 was above 400 nm. The critical aggregation concentrations (CAC) of the nanogels in distilled water were $<1 \times 10^{-1}$ mg/mL. The doxorubicine (DOX) loading efficiencies and loading contents of AC-HA_{LM} increased as the degree of acetylation increased, in particular, the loading efficiency of AC-HA_{LM}3 reached above 90%. AC-HA_{LM}3 nanogels showed IC_{50} at 1300 ng/mL of the DOX concentration against HeLa cells (with HA-binding receptors) similar to free DOX. For monitoring of specific interaction with a carcinoma cell line (HeLa cells with HA-binding receptors), AC-HA_{LM}3 was labeled with FITC and observed with a confocal microscope. HeLa cells were strongly luminated by interactions with fluorescence-labeled AC-HA_{LM}3 nanogels; however, this luminance was significantly decreased by competition inhibition of free HA. The results indicate that modified HA maintains the ability to interact with HA-binding receptors. The selective cytotoxicity and interaction of AC-HA_{LM} nanogels may help reduce side effects of anti-cancer drugs in clinical use.

Gupta and his co-worker prepared hydrogel nanoparticles of cross-linked pullulans with glutaraldehyde in order to develop a DNA carrier system, improving the gene loading efficacy, controlled release properties, biocompatibility and enhanced stability.

This study provided a method for enhancing the delivery of nucleic acid molecules to cells by encapsulating it inside the hydrogel pullulan nanoparticles. In this study, pullulan nanoparticles encapsulating pBUDLacZ plasmid was prepared inside the aqueous droplets of w/o microemulsions. Transmission electron microscopy (TEM) image showed that the particles are spherical in shape with size of 45 ± 0.80 nm diameter. Cell cytotoxicity studies as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay demonstrated that cells incubated with nanoparticles remained more than 100% viable at nanoparticle concentration as high as 1000 μg/ml. From scanning electron microscope images, it was observed by them that the nanoparticles were internalized and the cells exhibited vacuoles in the cell body due to nanoparticle internalization. Endocytosis of nanoparticles resulted in disruption of F-actin and β-tubulin cytoskeleton of human fibroblasts. The efficacy of transfection in vitro on HEK293 and COS-7 cells demonstrated cell type dependence, with COS cells having a higher gene expression. The β-gal expression in COS-7 cells by pullulan nanoparticle was comparable to commercially available Lipofectamine 2000. The results of this study were encouraging for the development of pullulan nanoparticles as an intracellular delivery system for drugs and genes (also, hydrophobized pullulan-based nanogels interact with molecular assemblies such as liposomes and oil–water emulsion). As a result, hydrophobized pullulan conjugates were used for targeting of drugs viz., metronidazole, nicotinic acid, sulfathilzole, mitoxantrone and epirubicin. Most of the cited paper in the field of hydrophobized pullulan reports the self-assembly of cholesterol-bearing pullulan as stable hydrogel nanoparticles in which pullulan is non-covalently bind by associating cholesteryl moieties is shown in (Fig. 2) (Gupta & Gupta, 2004).

6.5. *Insulinotropic activity of sulfonylurea/pullulan conjugate*

The in vitro long-term effect of a water-soluble sulfonylurea/pullulan conjugate (SUP) on insulinotropic activity and cell viability were investigated using rat pancreatic islets co-entrapped with SUP in conventional alginate-poly (l-lysine) microcapsules. The conjugate was synthesized by coupling a carboxylated glibenclamide derivative to a polysaccharide, pullulan (MW = 200,000). In vitro static experiment showed that sulfonylurea concentration in SUP over 50 mm was required to stimulate the rat islets. In a dynamic insulin secretion test, the microcapsules of islets with SUP regained the insulin secretion pattern comparable to that of free islets, while those without SUP showed impaired insulin secretion. The long-term (1 month) culture experiment demonstrated that the microcapsules of islets with SUP, with well-preserved morphology, presented higher insulin secretion level and better ability in responding to glucose changes than those without SUP (Sungwon, Su, Kun, Sung, & You, 2003).

Table 2
Recent advances of pullulan.

S. no.	Recent research	Advantages of pullulan	References
1	Fast disintegrating tablet using pullulan as diluent	Tablet hardness was found to increase without increasing the disintegrating time with high concentration of pullulan.	Patel, Chauhan, Patel, and Patel (2012)
2	Pullulan/silver nanoparticles composite nanospheres using electrospray techniques for antibacterial application	Controlled spherical structure by controlling the concentration of pullulan enhanced antibacterial activity.	Islam et al. (2011)
3	Self-assembled nanogels of hydrophobized pullulan	Size stability, micelles showed long term colloidal stability with nearly negative neutral charge.	Ferreira, Coutinho, and Gama (2011)
4	Pullulan acetate coated magnetic nanoparticles for hyperthermia	Nanoparticles have high magnetite content, good biocompatibility, good heating property in magnetic field, and have evident cellular uptake by tumor cells.	Gao et al. (2010)
5	Rapid dissolving films of cetirizine hydrochloride using pullulan as a film forming agent	Pullulan acted as rapid film forming agent.	Mishra and Amin (2011)
6	Preparation of ion-exchange membranes using pullulan as polymer matrix	Pullulan was used to prepare membranes.	Lebrun, Blanco, and Metayer (2005)

6.6. Pullulan as a polymer in formulation of carbon nanotubes (CNTs)

CNTs belong to the fullerene family of carbon allotropes with cylindrical shape. The unique physicochemical properties (Cui et al., 2010; Huang, Zhang, Xu, Bao, & Li, 2010) of CNTs with easy surface modification have led to a surge in the number of publications in this interesting field. Apart from their uses in the cellular imaging with diagnostic effects in nanomedicine (Bao, Tian, & Estrada, 2010; Chen et al., 2010) CNTs are promising drug carriers in the target drug delivery systems for cancer therapies. Pullulan is used as a polymer for solubilizing the CNTs. It is also for helical wrapping of linear or branched polysaccharides around the surface of CNT (Numata et al., 2005).

6.7. Pullulan as a film forming agent in preparation of oral thin films

Pullulan is widely used as a film forming agent in the preparation of variety of Oral thin films because of its various properties like it is impermeable to oxygen, non-hygroscopic and non-reducing. It is easily soluble in hot water and cold water to make clear and viscous solution and also has high adhesion and film forming abilities. The principal advantages of pullulan are that it is a nonionic polysaccharide and is blood compatible, biodegradable, non-toxic, non immunogenic, nonmutagenic and non carcinogenic (Priyanka, Iti, & Mohd, 2011). The development of a fast-dissolving film also provides an opportunity for a line extension in the market place; a wide range of drugs (e.g., neuroleptics, cardiovascular drugs, analgesics, antihistamines, antiasthmatics and drugs for erectile dysfunction) can be considered candidates for this dosage form.

6.8. Surface modification

Yet another promising application of this versatile polymer is the use in surface modification as evidenced by work by Hasuda et al. Photoreactive pullulan was prepared, the polymer was photoimmobilized on polymeric or organic surfaces, and its interactions with a protein and a cell type were investigated. The photoreactive pullulan was synthesized by a coupling reaction with 4-azidobenzonic acid. Surface modification was carried out in the presence or absence of a micropatterned photomask containing 100 μm transparent stripes with 150 μm gaps, making it easy to confirm the immobilization. By the micropatterning method, immobilization of the photoreactive pullulan on polystyrene, polyethylene, and silane-coupled glass was confirmed. Contact angles were measured on the unpatterned surfaces. Although the original surfaces have different contact angles, the contact angle on Az-pullulan-immobilized surface was the same on all surfaces. This result demonstrated that photoimmobilization completely covered the surface with Az-pullulan. Protein adsorption was investigated using fluorescently labeled albumin applied to the micropatterned surface: fluorescence microscopy demonstrated that adsorption was reduced on the pullulan-immobilized regions. Culture of RAW264 cells, derived from mouse leukemic monocytes, on the micropatterned surface for 22 h showed that cells did not adhere to the immobilized pullulan regions. In conclusion, photoreactive pullulan was covalently immobilized on various surfaces and tended to reduce interactions with proteins and cells (Hasuda, Kwon, Kang, & Ho, 2005).

6.9. Targeted drug delivery with magnetic nanoparticles by its surface modification

Targeted drug delivery with magnetic nanoparticles is possible with the use of external magnetic fields target the particle to the site of interest. Magnetic nanoparticles are also of interest in diagnostic imaging as well. But since the magnetic nanoparticles being hydrophobic gets easily destroyed or cleared from the circulation and these particles are also cytotoxic. Hydrophilic surface modification of these particles prolongs the half-life in the circulation. Gupta et al. coated prepared superparamagnetic iron oxide nanoparticles (SPION) and coated with pullulan (Pn-SPION). They studied the effect of pullulan coating on the cytotoxicity and the cellular uptake of the nanoparticles. The cytotoxicity studies were done on fibroblasts and it was observed that with uncoated particles (SPION) the cell death was 60% and with Pn-SPION there were no cytotoxic effects. Similarly cell adhesion test also showed that the attached cell number was decreased up to 64% in SPION but for pullulan coated it was comparable with the control cell population. The authors attribute the low toxicity of Pn-SPION to the hydrophilicity of pullulan. By transmission electron microscopy the cellular uptake of the particles was also established. These pullulan coated magnetic particles is thought to be useful for medical imaging like vascular compartment imaging, lymph node, receptor, perfusion and target specific imaging (Ajay & Mona, 2005).

6.10. As a mucoadhesive polymer in treatment of ocular disease

To improve the ocular bioavailability of drugs, numerous natural and synthetic viscosifying agents were added to the vehicle in order to increase the viscosity of the preparation, to reduce the

drainage rate and subsequently to improve the therapeutic efficacy. Amongst these polymers, Pullulan is used as mucoadhesive ophthalmic vehicle. Also, in the case of polysaccharides, the formation of macromolecular ionic complexes with drugs improved the bioavailability and lengthened the therapeutic effect when compared to drug solutions (Annick, 2005).

7. Recent advances of pullulan

Investigated recent advances of pullulan are described in tabular format in Table 2.

8. Conclusion

Pullulan has gained a lot of attention in the past few decades due to its unique properties. Pullulan is an edible and biopolysaccharide with numerous applications in the field of food and pharmaceutical industries. The unique property of pullulan is due to its glycosidic linkage. Pullulan is synthesized by fermentation of coconut by-products, beet molasses, agro-industrial waste. Pullulan can be easily derivatized by means of chemical reaction. Pullulan has important application in the field of biomedical and pharmaceutical field viz., tissue engineering and grafting. Pullulan has been used for liver and tumor target delivery of drug. Pullulan has the application in the field of targeting of drug to liver and cancer cells. Pullulan has occupied a niche area in food and pharmaceutical field.

Acknowledgement

The authors are highly thankful to SSR College of Pharmacy, Silvassa for providing all the necessary support and the essential library information resource.

References

Ajay, G., & Mona, G. (2005). Cytotoxicity suppression and cellular uptake enhancement of surface modified magnetic nanoparticles. *Biomaterials*, *26*, 1565–1573.
Alban, S., Schauerte, A., & Franz, G. (2002). Anticoagulant sulfated polysaccharides. Part I. Synthesis and structure–activity relationships of new pullulan sulfates. *Carbohydrate Polymers*, *47*(3), 267.
Annick, L. (2005). The use of mucoadhesive polymer in ophthalmic drug delivery system. *Advanced Drug Delivery Review*, *57*(11), 1595–1639.
Bao, C., Tian, F., & Estrada, G. (2010). Improved visualisation of internalised carbon nanotubes by maximising cell spreading on nanostructured substrates. *Nano Biomedicine and Engineering*, *2*(4), 201–207.
Bataille, I., Huguet, J., Muller, G., Mocanu, G., & Caprov, A. (1997). Associative behaviour of hydrophobically modified carboxymethyl pullulan derivative. *International Journal of Biological of Macromolecules*, *20*(3), 179.
Bataille, I., Meddahi-Pelle, A., Visage, C. L., Letourneur, D., & Chaubet, F. (2011). Pullulan for biomedical use. In Valentin popa (Ed.), *Polysaccharides in medicinal and pharmaceutical application* (pp. 145–182). iSmithers Rapra Publishing (Chapter 4).
Bender, H., Lechmann, J., & Wallenfells, K. (1959). Pullulan in extracellular glucans von *Pullularia pullulans*. *Biochemica Biophysica Acta*, *36*, 309–316.
Bishwambhar, M., Suneetha, V., & Ramalingam, C. (2011). An overview of characterization and optimization of pullulan producing microorganism. *South Asian Journal of Experimental Biology*, *1*, 147–151.
Campbell, B. S., Siddique, A. B. M., McDougall, B. M., & Seviour, R. J. (2004). Which morphological forms of the fungus *Aureobasidium pullulans* are responsible for pullulan production? *FEMS Microbiology Letters*, *232*, 225–228.
Carolan, G., Catley, B. J., & McDougal, F. G. (1983). The location of tetrasaccharide units in pullulan. *Carbohydrate Research*, *114*, 237–243.
Cheek, K. (1990). Determination of polymer–polymer miscibility by viscometry. *European Polymer Journal*, *26*, 423–426.
Chen, D., Wu, X., Wang, J., Han, B., Zhu, P., & Peng, C. H. (2010). Morphological observation of interaction between PAMAM dendrimer modified single walled carbon nanotubes and pancreatic cancer cells. *Nano Biomedicine and Engineering*, *2*(4), 60–65.
Chi, Z., Wang, F., Chi, Z., Yue, L., Liu, G., & Zhang, T. (2009). Bioproducts from *Aureobasidium pullulans*, biotechnologically important yeast. *Applied Microbiology and Biotechnology*, *82*, 793–804.
Cleland, J. L., Hedgepeth, C., & Wang, D. I. C. (1992). Polyethylene glycol enhanced refolding of bovine carbonic anhydrase B. *Journal of Biological Chemistry*, *267*, 13327–13330.
Cui, D., Zhang, H., Sheng, J., Wang, Z., Toru, A., He, R., et al. (2010). Effects of CdSe/ZnS quantum dots covered multi-walled carbon nanotubes on murine embryonic stem cells. *Nano Biomedicine and Engineering*, *2*(4), 236–244.
Degeest, B., & Vuyst, L. D. (2000). Correlation of activities of the enzymes α-phosphoglucomutase, UDP-galactose 4-epimerase and UDP-pyrophosphorylase with exopolysaccharide biosynthesis by *Streptococcus thermophilus* LY03. *Applied and Environmental Microbiology*, *66*, 3519–3527.
Duan, X., Chi, Z., Wang, L., & Wang, X. (2008). Influence of different sugars on pullulan production and activities of α-phosphoglucose mutase, UDPG-pyrophosphorylase and glucosyltransferase involved in pullulan synthesis in *Aureobasidium pullulans* Y68. *Carbohydrate Polymers*, *73*(4), 587–593.
Ferreira, S. A., Coutinho, P. J. G., & Gama, F. M. (2011). Synthesis and characterization of self-assembled nanogels made of pullulan. *Materials*, *4*, 601–620.
Fundueanu, G., Constantin, M., Mihai, D., Bortolotti, F., Cortesi, R., & Ascenzi, P. (2003). Pullulan-cyclodextrin microspheres. A chromatographic approach for the evaluation of the drug–cyclodextrin interactions and the determination of the drug release profiles. *Journal of Chromatography B*, *791*, 407–419.
Gao, F., Cai, Y., Zhou, J., Xie, X., Ouyang, W., Zhang, Y., et al. (2010). Pullulan acetate coated magnetite nanoparticles for hypothermia: Preparation, characterization and in-vitro experiments. *Nano Research*, *3*, 23–31.
Gao, J., Yu, J., Wang, W., Chang, L., & Tian, R. (1998). Graft copolymerization of starch – An initiated by potassium permanganate. *Journal of Applied Polymer Science*, *68*(12), 1965–1972.
Gheorghe, F., Marieta, C., & Paolo, A. (2008). Preparation and characterization of pH- and temperature-sensitive pullulan microspheres for controlled release of drugs. *Biomaterials*, *29*, 2767–2775.
Goksungur, Y., Ucan, A., & Guvenc, U. (2004). Production of pullulan from beet molasses and synthetic medium by *Aureobasidium pullulans*. *Turkish Journal of Biology*, *28*, 23–30.
Gupta, M., & Gupta, A. K. (2004). Hydrogel pullulan nanoparticles encapsulating pBUDLacZ plasmid as an efficient gene delivery carrier. *Journal of Controlled Release*, *99*, 157–166.
Hasuda, H., Kwon, O. H., Kang, I.-K., & Ho, Y. (2005). Synthesis of photoreactive pullulan for surface modification. *Biomaterials*, *26*, 2401–2406.
Henni-Silhadi, W., Deyme, M., Hoyos, M. R., Cerf, D. L., Picton, L., & Rosilio, V. (2008). Influence of alkyl chains length on the conformation and solubilization properties of amphiphilic carboxymethyl pullulans. *Colloid and Polymer Science*, *286*, 1299–1305.
Hosseinkhani, H., Aoyama, T., Ogawa, O., & Tabata, Y. (2002). Liver targeting of plasmid DNA by pullulan conjugation based on metal coordination. *Journal of Controlled Release*, *83*, 287–302.
Huang, P., Zhang, C., Xu, C., Bao, L., & Li, Z. (2010). Preparation and characterization of near-infrared region absorption enhancer carbon nanotubes hybrid materials. *Nano Biomedicine and Engineering*, *2*(4), 225–230.
Imshenetskii, A. A., Kondrateva, T. F., Kudryashev, L. I., Yarovaya, S. M., Smutko, A. N., & Alekseeva, G. S. (1983). A comparative study of pullulans synthesized by strains of *Pullularia (Aureobasidium) pullulans* of differing levels of ploidy. *Journal of Microbiology*, *52*, 816–820.
Islam, Md. S., Islam, Md. A., Sarker, M., Karim, Md. M., Masum, S., & Yeum, J. H. (2011). Fabrication of pullulan/silver nanoparticle composite nanospheres using electrospray technique for antibacterial applications. *International Journal of Basic and Applied Sciences*, *11*(1), 59–69.
Israilides, C. J., Smith, A., & Bambalov, G. (1993). Production of pullulan from agro-industrial wastes. In *Proceedings of the sixth European congress on biotechnology, Frienz, Italy* (p. 362).
Israilides, C. J., Smith, A., Harthill, J. E., Barnett, C., Bambalov, G., & Scanlov, B. (1998). Pullulan content of the ethanol precipitate from fermented agro-industrial wastes. *Applied Microbiology and Biotechnology*, *49*, 613–617.
Israillides, C. J., Scanlon, B., Smith, A., Jumel, K., & Harding, S. E. (1994). Characterization of pullulans produced from agro-industrial wastes. *Carbohydrate Polymers*, *25*, 203–309.
Israilides, C. J., Vlyssides, A. G., Mouraferi, V. N., & Karvouni, G. (1977). Olive oil waste water treatment with the use of an electrolysis system. *Bioresource Technology*, *61*, 163–170.
Kimoto, T., Shibuya, T., & Shiobara, S. (1997). Safety studies of a novel starch, pullulan: Chronic toxicity in rats and bacterial mutagenecity. *Food and Chemical Toxicology*, *35*, 323–329.
Kin, E. J., Cho, S. H., & Yuk, S. H. (2001). Polymeric microspheres composed of pH/temperature-sensitive polymer complex. *Biomaterials*, *22*, 2495–2499.
Kondratyeva, T. F. (1981). Production of the polysaccharide pullulan by *Aureobasidium (Pullularia) pullulans*. *Uspechi Microbiology*, *16*, 175–193.
Kurkuri, M. D., & Aminabhavi, T. M. (2004). Poly (vinyl alcohol) and poly (acrylic acid) sequential interpenetrating network pH-sensitive microspheres for the delivery of diclofenac sodium to the intestine. *Journal of Controlled Release*, *96*, 9–20.
Lebrun, L., Blanco, J. F., & Metayer, M. (2005). Preparation of ion-exchange membranes using pullulan as polymer matrix. *Carbohydrate Polymer*, *61*, 1–4.
Lee, J. H., Kim, J. H., Zhu, I. H., Zhan, X. B., Lee, J. W., Shin, D. H., et al. (2001). Optimization of conditions for the production of pullulan and high molecular weight pullulan by *Aureobasidium pullulans*. *Biotechnology Letters*, *23*, 817–820.
Legros, M., Dulong, V., Picton, L., & Cerf, D. L. (2008). Self-organization of water soluble and amphiphile cross linked carboxymethyl pullulan. *Polymer Journal*, *40*(12), 1132.
Li, H., Chi, Z., Wang, X., Duna, X., Ma, L., & Gao, L. (2007). Purification and characterization of extracellular amylase from the marine yeast *Aureobasidium pullulans*

N13d and its raw potato starch digestion. *Enzyme and Microbial Technology*, *40*, 1006.
Mahner, C., Lechner, M. D., & Nordmeier, E. (2001). Synthesis and characterization of dextran and pullulan sulphate. *Carbohydrate Research*, *331*(2), 203.
Mishra, B., Vuppu, S., & Rath, K. (2011). The role of microbial pullulan, a biopolymer in pharmaceutical approaches: A review. *Journal of Applied Pharmaceutical Sciences*, *1*(6), 45–50.
Mishra, R., & Amin, A. (2011). Formulation and characterization of rapidly dissolving films of cetrizine hydrochloride using pullulan as film forming agent. *Indian Journal of Pharmaceutical Education and Research*, *45*(1), 71–77.
Mocanu, G., Mihai, D., Le Clerf, D., Picton, L., & Muller, G. (2004). Synthesis of new associative gel microspheres from carboxymethyl pullulan and their interactions with lysozyme. *European Polymer Journal*, *40*, 283–289.
Na, K., Lee, D. H., Hwang, D. J., Park, H. S., Lee, K. H., & Bae, Y. H. (2006). pH-sensitivity and pH dependent structural change in polymeric nanoparticles of poly (vinyl sulfadimethoxine)-deoxycholicacidconjugate. *European Polymer Journal*, *42*, 2581–2588.
Nobuyuki, M., Takao, E., Michiko, O., Yasuhiko, I., & Kazunari, A. (2005). Hybrid nanogels with physical and chemical cross-linking structures as nanocarriers. *Macromolecular Bioscience*, *5*, 710–716.
Nomura, Y., Ikeda, M., Nozomi, M., Yamaguchi, N., Aoyama, Y., & Akiyoshi, K. (2003). Protein refolding assisted by self-assembled nanogels as novel artificial molecular chaperone. *FEBS Letters*, *553*(3), 271–276.
Numata, M., Asai, M., Kaneko, K., Bae, A. H., Hasegawa, T., Sakurai, K., et al. (2005). Inclusion of cut and as-grown single-walled carbon nanotubes in the helical superstructure of schizophyllan and curdlan (N-13-glucans). *Journal of American Chemical Society*, *127*, 5875–5884.
Patel, D., Chauhan, M., Patel, R., & Patel, J. (2012). Optimization of fast disintegration tablets using pullulan as diluent by central composite experimental design. *Journal of Pharmacy and Bioallied Sciences*, *4*(5), 86–87.
Priyanka, N., Iti, C., & Mohd, Y. (2011). Insights into polymers: Film formers in mouth dissolving films. *Drug Invention Today*, *3*(12), 280–289.
Rekha, M. R., & Sharma, C. P. (2009). Blood compatibility and in-vitro transfection studies on cationically modified pullulan for liver cell targeted gene delivery. *Biomaterials*, *30*, 6655–6664.
Roukas, T., & Biliaderis, C. (1995). Evaluation of carbo pod as a substrate for pullulan production by *Aureobasidium pullulans*. *Applied Biochemistry and Biotechnology*, *55*, 27–44.
Saha, B. C., & Bothast, R. J. (1993). Starch conversion by amylases from *Aureobasidium pullulans*. *Journal of Industrial Microbiology*, *12*, 413–416.
Scomparin, A., Salmosa, S., Bersani, S., Satchi-Fainaro, R., & Caliceti, P. (2011). Novel folated and non-folated pullulan bioconjugates for anticancer drug delivery. *European Journal of Pharmaceutical Science*, *42*, 547–558.
Shabtai, Y., & Mukmenev, J. (1995). Enhanced production of pigment-free pullulan by a morphogenetically arrested *Aureobasidium pullulans* (ATCC 42033) in two stage fermentation with shift from soyabean oil to sucrose. *Applied Microbiology and Biotechnology*, *43*, 595–603.
Shin, Y. C., Kim, Y. H., Lee, H. S., Cho, S. J., & Byum, S. M. (1989). Production of exopolysaccharide pullulan from inulin by a mixed culture of *Aureobasidium pullulans* and *Kluyveromyces fragilis*. *Biotechnology and Bioengineering*, *33*, 129–133.
Shingel, K. I. (2004). Current knowledge on biosynthesis, biological activity, and chemical modification of the exopolysaccharide, pullulan. *Carbohydrate Research*, *339*, 447–460.
Shingel, K. I., & Petrov, P. T. (2002). Behaviour of γ-ray-irradiated pullulan in aqueous solutions of cationic (cetyltrimethylammoniumhydroxide) and anionic (sodium dodecyl sulfate) surfactants. *Colloid and Polymer Science*, *280*, 176–182.
Simon, I., Caye-Vaugien, C., & Bouchonneau, M. (1993). Relation between pullulan production, morphological state and growth conditions in *Aureobasidium pullulans*: New observations. *Journal of General Microbiology*, *139*, 979–985.
Souguir, Z., Roudesli, S., Picton, L., Cerf, D., & About-Jaudet, E. (2007). Novel cationic and amphiphilic pullulan derivatives I: Synthesis and characterization. *European Polymer Journal*, *43*, 4940–4950.
Sun, Z., Wang, W., & Feng, Z. (1992). Criterion of polymer–polymer miscibility determined. *European Polymer Journal*, *51*, 1259.
Suneetha, V., Sindhuja, K. V., & Sanjeev, K. (2010). Screening, characterization and optimization of pullulan producing microorganisms from plant leaves in chitoor district. *Asian Journal of Microbiology Biotechnology and Environmental Sciences*, *12*, 149–155.
Sungwon, K., Su, Y. C., Kun, N., Sung, W. K., & You, H. B. (2003). Insulinotropic activity of sulfonylurea/pullulan conjugate in rat islet microcapsule. *Biomaterials*, *24*, 4843–4851.
Thirumavalavan, K., Manikkadan, T. R., & Dhanasekar, R. (2009). Pullulan production from coconut by-products by *Aureobasidium pullulans*. *African Journal of Biotechnology*, *8*(2), 254–258.
Tsijisaka, Y., & Mitsushashi, M. (1993). In R. Whistler, & J. N. BeMiller (Eds.), *Pullulan in industrial gum: Polysaccharides and their derivatives* (pp. 447–460). San Diego: Academic.
Wallenfels, K., Bender, H., Keilich, G., & Bechtler, G. (1961). On pullulan, the glucan of the slime coat of *Pullularia pullulans*. *Angew Chem*, *73*, 245–246.
West, T. P., & Strohfus, B. (2001). Polysaccharide production by immobilized *Aureobasidium pullulans* cells in batch bioreactors. *Microbiology Research*, *156*, 285–288.
Wooram, P., Kyoung, S. K., Byoung-chan, B., Young-Heui, K., & Kun, N. (2010). Cancer cell specific targeting of nanogels from acetylated hyaluronic acid with low molecular weight. *European Journal of Pharmaceutical Sciences*, *40*, 367–375.
Xi, K., Tabata, Y., Uno, K., Yoshimoto, M., Kishida, T., Sokawa, Y., et al. (1996). Liver targeting of interferon through pullulan conjugation. *Pharmaceutical Research*, *13*, 1846–1850.
Youssef, F., Biliaderis, C. G., & Roukas, T. (1998). Enhancement of pullulan production by *Aureobaisdium pullulans* in batch culture using olive oil and sucrose as carbon source. *Applied Biochemistry and Biotechnology*, *74*, 13–30.
Zheng, W., Campbell, B. S., McDougall, B. M., & Seviour, R. (2008). Effects of melanin on the accumulation of exopolysaccharides by *Aureobasidium pullulans* grown on nitrate. *Bioresource Technology*, *99*, 7480.