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Synthesis and Characterization of Biopolymers Pullunan

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Abstract:

Pullulan is a natural exopolysaccharide with many useful characteristics. However, pullulan is more costly than other exopolysaccharides, which limits its effective application. The purpose of this study was to adopt a novel mixed-sugar strategy for maximizing pullulan production, mainly using potato starch hydrolysate as a low-cost substrate for liquid-state fermentation by *Aureobasidium pullulans*. Based on fermentation kinetics evaluation of pullulan production by *A. pullulans* 201253, the pullulan production rate of *A. pullulans* with mixtures of potato starch hydrolysate and sucrose was $0.212h^{-1}$, which was significantly higher than those of potato starch hydrolysate alone and mixtures of potato starch hydrolysate, glucose, and fructose (potato starch hydrolysate:glucose:fructose with $100gL^{-1}$ total carbon source. The results suggest that mixtures of potato starch hydrolysate and sucrose could promote pullulan synthesis and possibly that a small amount of sucrose stimulated the enzyme responsible for pullulan synthesis and promoted effective potato starch hydrolysate conversion effectively. Thus, mixed sugars in potato starch hydrolysate and sucrose fermentation might be a promising alternative for the economical production of pullulan.

Keywords: Pullulan, biopolymer, sucrose, characterization

Introduction: A natural polymer, produced by *Aureobasidium pullulans* (*A. pullulans*) using various carbon sources. It has several applications, primarily in the pharmaceutical and food industries. Pullulan is biodegradable in nature. Pullulan is a linear polysaccharide, and its structural formula represented as a sequence of panoses connected by α-(1→4)- linkages (fig. 1). Alternatively, it can be described as having maltotriosyl [α-(1→4) Glcp- α-(1→4) Glcp] units connected by α-(1→6)-linkages[1-3].This polymer use in medical field is increasing contemporarily. In comparison to dextran, pullulan utilization rate in serum is faster. This polymer degradation index is 0.7 after 48 h while for dextran it is only 0.05[10].

Pullulan films have been used for various food applications. Pullulan film is commercially used in the US in Listerine PocketPacks® oral care strips. Pullulan has several potential commercial applications, primarily in the food and pharmaceutical industries. It is edible, odourless and flavourless, thus it can be used as food additive to reduce blemishes, improve appearance and increase shelf life. It has good adhesive property, low viscous in nature and making it suitable for use in various industrial applications. This polymer can be incorporated for improving dispersibility and consistency[4-7].

Material and Methods:

All the materials required for the research study such as strains and chemicals were purchased from the various companies.

Pullulan Production Method:

Preliminary Screening: The strains were inoculated and incubated on growth medium plate containing Congo red and trypan blue. The dye Congo red specifically binds with EPS producing organisms and trypan blue is specific for α-glucan type of polysaccharides. Medium containing 0.5% yeast extract, 1% glucose, 0.005% Congored / trypan blue and 2% agar, pH 7.2 was used to detect positive strains[8].

The colony that showed an intense red/blue colour on the plates was selected for further study**.**

Inoculum Development: Plates containing test strains mentioned in table 7 were incubated at suitable temperature depending on the strain (Appendix). Test strains of fungi were exposed to black light to stimulate sporulation. The cultures were allowed to grow for 3 days to induce spore formation. Cells were harvested by flooding the culture plates with sterile distilled water to achieve a 10^4 -10^6 cells/ml stock[9].

Table 1: Preliminary screening of test strains and cultivation conditions for pullulan production

Sr. No.	Test strains	Media*	Temperature (°C)
1.	Aureobasidium pullulans NCIM 1049 (Reference strain)	PDA	28
2.	Aureobasidium mausonii NCIM 1226 (ATCC 3726)	PDA	28
3.	Rhodotorula glutinis NCIM 3168	MGYP	28
4.	Rhodotorula graminis NCIM 3426	MGYP	28
5.	Rhodotorula marina NCIM 3415	MGYP	28
6.	Rhodotorula minuta NCIM 3359	MGYP	28
7.	Rhodotorula rubra NCIM 3171	MGYP	28
8.	Rhodotorula species NCIM 3560	MGYP	28

Inoculum Medium Preparation: The prepared stock was directly inoculated into the seed medium contained (g/l), sucrose, 50.0; yeast extract, 2.0; K_2HPO_4, 5.0; $(NH_4)_2SO_4$, 0.6; $MgSO_4$ $7H_2O$, 0.2; NaCl, 1.0 and distilled water l L. The medium was autoclaved at 121°C for 15 min and the initial pH was adjusted to 5.0 and 7.4 for fungi and bacteria respectively. Inoculum medium was incubated at 28°C for 48 h with shaking at 150 rpm[10].

Determination of Growth Kinetics: The growth curve kinetics and quantification of extractable polymer were determined by culturing the strain in the fermentation medium. This was done by inoculating 10% of inoculum in 500-ml flask. The mycelial dry weight was determined by filtration and drying the biomass at 65°C; extractable polymers were determined after 24, 48, 72, 96 and 120 h of growth as described above[11].

Optimization of Pullulan Production: The yield of the polymers was enhanced by optimizing various factors

In this research single point optimization technique was used to optimize the media for pullulan production by the selected test strains[12-15].

Inoculum Size Optimization: Inoculum size also affects the production of the pullulan in fermentation processes. In this research the media was inoculated using an inoculum size varying from 2% to 14% (v/v). All other parameters were maintained constantly[16-18].

Medium Optimization: The carbon and nitrogen source requirements for the various test strains was studied and optimized.

Nitrogen Source Optimization: The carbon source i.e. glucose and mineral sources used earlier was kept constant and effect of various nitrogen sources, namely corn steep liquor (CSL), ammonium sulphate, yeast extract, urea, peptone and sodium nitrite were studied[19-21].

Carbon Source Optimization: The nitrogen source and mineral sources mentioned in the fermentation medium was kept constant and various carbon sources, namely fructose, maltose, sucrose and lactose were studied[22-23].

Result and Discussion:

Strain identification: The initial screening was performed with Congo red. Based on the staining pattern 19 positive strains were identified in this study for further production of pullulan.

Pullulan Identification in Fungi: Among the tested strains for pullulan production, *A.pullulans*, *A.mausonii* NCIM 1226, *R.glutinis* NCIM 3168 *and R.minuta* NCIM 3359 were selected for

further study based on the colour intensity in the growth medium plate containing Congo red and trypan blue . Shake flask culture method in duplicate was adopted for pilot study with the selected positive strains for its production subsequently. The isolated polymer was analysed for yield and **(a)** **(b)**

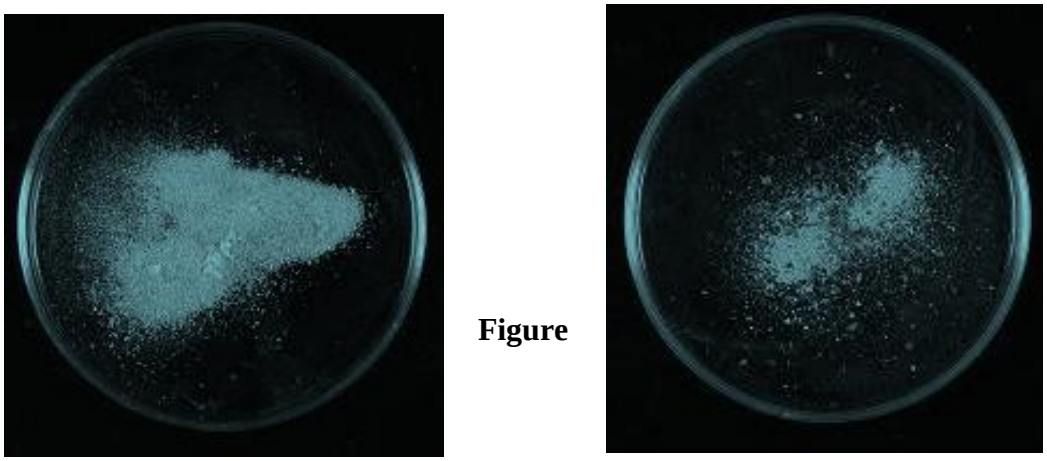

Figure 1: (a) Commercial Pullulan, (b) Pullulan from *A.mausonii*

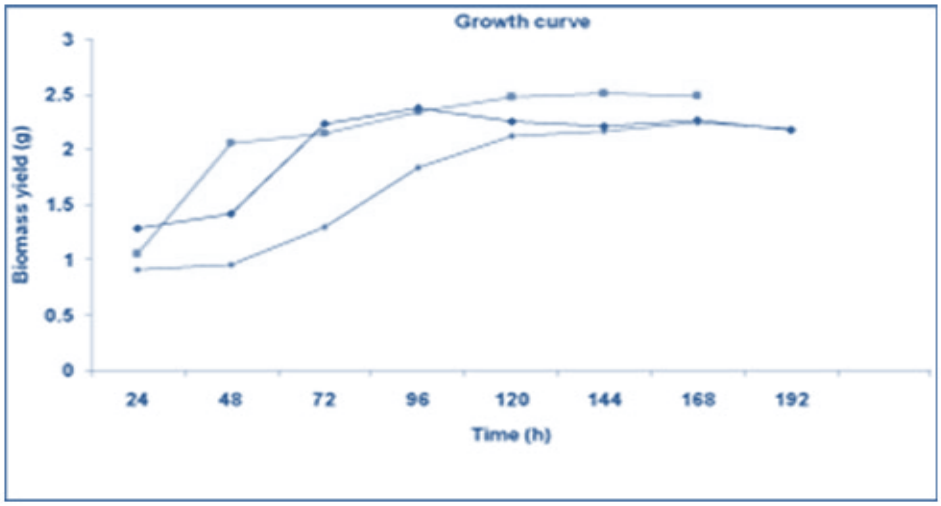

Figure 2: Growth curve of *A.mausonii, R.glutinis and R. minuta*

Table 2: Weight of Biomass, Extractable Pullulan *Aureobasidium mausonii* NCIM 1226

Sr. No	Time (h)	Weight of Biomass(g)	Weight of Pullulan (g)
01	24	2.29 ± 0.03	1.19 ± 0.03
02	48	2.92 ± 0.04	1.92 ± 0.04
03	72	3.53 ± 0.05	2.56 ± 0.05
06	96	4.57 ± 0.02	3.37 ± 0.02
07	120	4.21 ± 0.07	3.14 ± 0.07
04	144	3.11 ± 0.02	2.18 ± 0.02
05	168	2.26 ± 0.19	1.82 ± 0.19

Values are mean ± S.E.M (n=3)

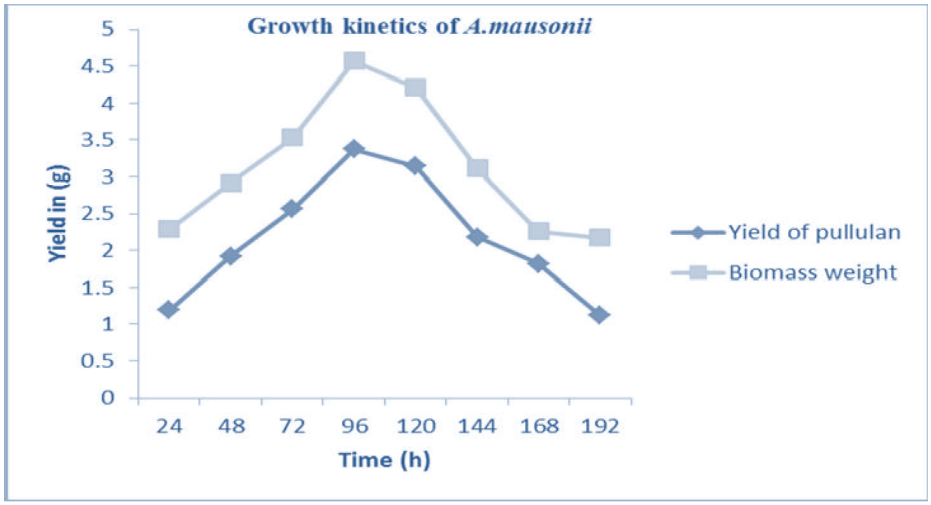

Figure 3: Weight of Biomass, Extractable Pullulan *Aureobasidium mausonii* NCIM 1226

Table 3: Weight of Biomass, Extractable Pullulan *Rhodotorula glutinis* NCIM 3168

Sr. No.	Time (h)	Weight of Biomass (g)	Weight of Pullulan (g)
01	24	1.58 ± 0.44	1.02 ± 0.41
02	48	2.14 ± 0.28	1.12 ± 0.62
06	72	3.35 ± 0.58	2.24 ± 0.47
03	120	4.28 ± 0.74	2.95 ± 0.86
05	96	4.01 ± 0.86	2.58 ± 0.74
07	144	3.45 ± 0.12	1.98 ± 0.63
04	168	2.91 ± 0.54	1.57 ± 0.45
08	192	1.98 ± 0.91	0.96 ± 0.55

Values are mean ± S.E.M (n=3)

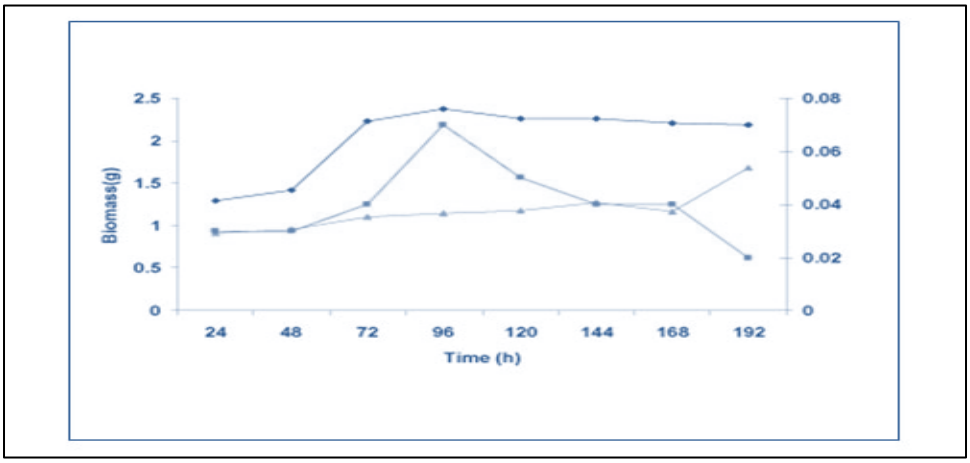

Figure 4: Weight of Biomass, Extractable Pullulan *Rhodotorula glutinis* NCIM 3168

Table 4: Weight of Biomass, Extractable Pullulan *R. minuta* NCIM 3359

Sr. No.	Time (h)	Weight of Biomass (g)	Weight of Pullulan (g)
01	24	1.61 ± 0.58	0.95 ± 0.25
02	48	2.35 ± 0.61	1.16 ± 0.48
03	72	3.07 ± 0.92	1.89 ± 0.98
04	96	4.17 ± 0.57	2.41 ± 0.54
05	120	3.85 ± 0.82	2.02 ± 0.68
06	144	3.15 ± 0.42	1.68 ± 0.84
07	168	2.57 ± 0.36	1.45 ± 0.18
08	192	2.02 ± 0.28	1.21 ± 0.24

Values are mean ± S.E.M (n=3)

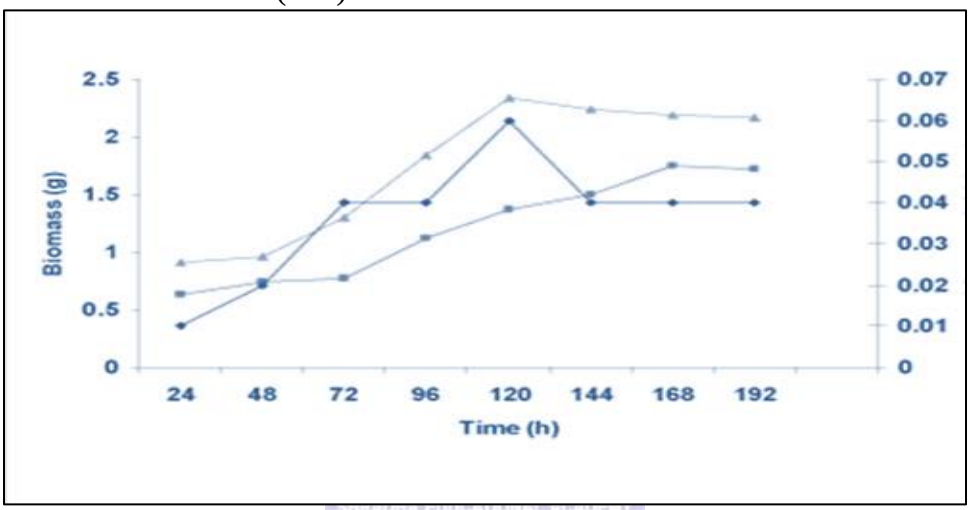

Figure 5: Weight of Biomass, Extractable Pullulan *R. minuta* NCIM 3359

Maximum yield of polymer was obtained with *R. minuta* NCIM 3359 (2.41 ± 0.54) on fourth day after which decrease in yield of polymer and biomass was observed. The presence of pigment was observed. In this research, the pigment was removed by using activated charcoal (1%). The reason behind decreased yield of polymer with *Rhodotorula sp.* may be due to the additional purification step involved in the removal of pigments produced during fermentation. This interpretation was based on earlier report indicating adherence of pullulan to the charcoal used for purification and there by its loss during filtration.

The main objective of this research was to identify new test strains that produce pullulan economically in large amounts without co production of pigment by submerged fermentation. From the preliminary screening six new strains were identified with possible pullulan production. Among the six strains only three strains produced recoverable amount of polymer from the fermentation broth among the three strains only one strain (*A.mausonii* NCIM 1226) produced a pigment free pullulan of considerable yield than the other strains. The pigment free production of pullulan with this fungus could be adopted as strain improvement method after various screening methods helps in the isolation of hyper producing *A.mausonii* NCIM 1226 strain. Hence further research was principally focussed with *A.mausonii* NCIM 1226.

Medium Optimization with Relation to Chitosan Production:

Polymer production using *A. mausonii* NCIM 1226, *R. glutinis* NCIM 3168 *and R.minuta* NCIM 3359 was affected based on the source of nitrogen in the production medium. Screening of

nitrogen source namely CSL, arginine, yeast extract, urea, peptone and sodium nitrite were studied using glucose as a carbon source at suitable pH with 250 rpm and the polymer was isolated after 96 h of fermentation.

The best yield of pullulan was observed with *A.mausonii* NCIM 1226 in the fermentation media containing urea as a nitrogen source (3.98 g) CSL (3.78 g) followed by sodium nitrite (3.61g). There was not much difference in yield of polymer with regard to the use of arginine and peptone (3.41 g and 3.48 g) as a nitrogen source. Less yield of polymer (2.78 g) was observed in the medium containing yeast extract.

The best yield of pullulan was observed with *R.glutinis* NCIM 3168 in the fermentation media containing yeast extract as a nitrogen source (2.88 g). This was followed by sodium nitrite (2.71 g) and urea (2.51 g). There was not much difference in yield of polymer with regard to CSL and Urea (2.25 g and 2.14 g). Yield of polymer was reduced with peptone as a nitrogen source in the medium (1.24 g).

The highest yield of pullulan was observed with *R.minuta* NCIM 3359 in the fermentation media containing sodium nitrite (2.56 g) and urea (2.51 g). There was not much difference in yield of polymer with peptone (2.24 g), yeast extract (2.18 g), CSL (2.12 g) and arginine (1.96 g) respectively. Yield of polymer was reduced when urea was used as a nitrogen source in the medium (1.74 g).

Apart from the yield of polymer the nitrogen source also affects on the MW[123]. Numerous research works on *A.pullulans* have studied the influence of nitrogen source on the yield of polymer production.

Table 5: Comparison of Effect of Different Carbon Sources on Pullulan Yield

Strains	**Carbon Source**					**Nitrogen Source**	**Mineral Salts**	**Yield of Pullulan (n=2)**
	Starch	Fructose	Maltose	Sucrose	Lactose	Peptone	K_2HPO_4, $MgSO_4$, $(NH4)_2SO_4$, NaCl, Soyabean	
A.mausoniiNCIM 1226	√					Urea	√	2.54 ± 0.11
		√					√	2.98 ± 0.01
			√				√	3.14 ± 0.21
				√			√	3.28 ± 0.18
					√		√	2.55 ± 0.41
R. glutinis NCIM 3168	√					Sodium Nitrite	√	1.98 ± 0.15
		√					√	2.06 ± 0.31
			√				√	2.28 ± 0.33
				√			√	2.96 ± 0.41
					√		√	2.78 ± 0.15
R.minuta	√					Sodium	√	1.54 ±

NCIM 3359						Nitrite		0.64
		√					√	2.10 ± 0.24
			√				√	2.12 ± 0.34
				√			√	1.86 ± 0.11
					√		√	2.44 ± 0.27

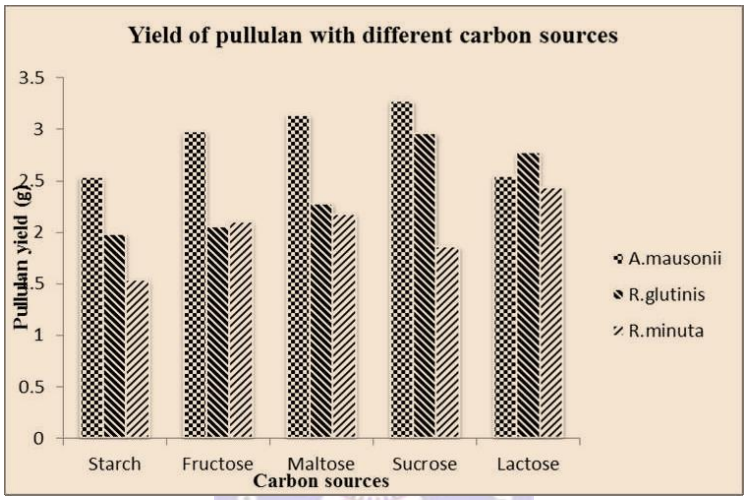

Figure 6: Comparison of Effect of Different Carbon Sources on Pullulan Yield

Effect of Carbon Source on Pullulan Production:

To produce cost effective pullulan, the effect of different easily available carbon sources was evaluated using the pH and nitrogen source optimized earlier in this study. The effect of different carbon sources namely glucose, starch, maltose, sucrose and lactose on pullulan yield was studied at pH 5.5 (*A.mausonii* NCIM 1226) and 6.5 (*Rhodotorula sp.*). Urea was used as a nitrogen source with *A.mausonii* NCIM 1226, sodium nitrite was used with *R.glutinis* NCIM 3168 and *R.minuta* NCIM 3359 respectively, as with these nitrogen sources the best yield was observed. The polymer was isolated at 96 h. The FT-IR spectra of the isolated polymers matched with reference polymer.

Table 6: Comparison of Effect of Different Nitrogen Sources on Pullulan Yield

Strains	**Nitrogen Source**						**Carbon Source**	**Mineral Salts**	**Yield of Pullulan (n=2)**
	Peptone	**CSL**	**Urea**	**Arginine**	**Sodium Nitrite**	**Yeast Extract**	**Glucose**	K_2HPO_4, $MgSO_4$, $(NH4)_2SO_4$, NaCl, Soyabean	
*A.mausonii*NCIM 1226	√						√	√	3.48 ± 0.25
		√					√	√	3.78 ± 0.34
			√				√	√	3.98 ± 0.18
				√			√	√	3.41 ± 0.01
					√		√	√	3.61 ± 0.47
						√	√	√	2.78 ± 0.51
R. glutinis	√						√	√	1.24 ± 0.21
		√					√	√	2.25 ± 0.35

NCIM 3168			√				√	√	2.51 ± 0.47
				√			√	√	2.14 ± 0.11
					√		√	√	2.71 ± 0.52
						√	√	√	2.88 ± 0.35
R.minuta NCIM 3359	√						√	√	2.24 ± 0.78
		√					√	√	2.12 ± 0.23
			√				√	√	1.74 ± 0.54
				√			√	√	1.96 ± 0.91
					√		√	√	2.56 ± 0.37
						√	√	√	2.18 ± 0.29

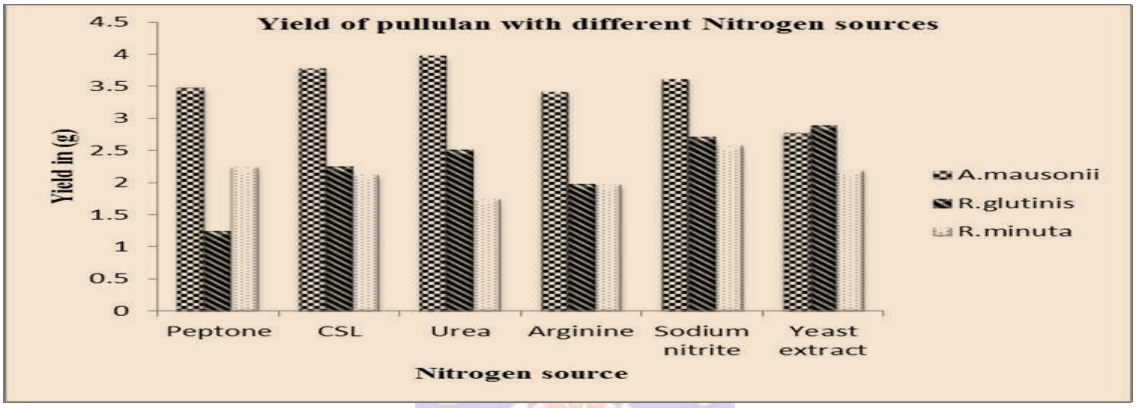

Figure 7: Comparison of Effect of Different Nitrogen Sources on Pullulan Yield

The basic medium containing glucose as a carbon source produced the best yield with the strain *A. mausonii* NCIM 1226 (3.37± 0.02). Sucrose (2.96 ± 0.41) and lactose (2.44 ± 0.27) were found to produce the best yield of pullulan with the *Rhodotorula sp*. In this research, decreased yield of polymer was observed with all the tested strains when starch containing fermentation medium was used. This may be due to the longer biosynthetic pathway involved in the conversion of starch and other carbon sources to UDP-glucose, an important precursor for pullulan synthesis[8]. The basic fermentation medium with glucose produced higher amount of polymer than the tested carbon sources consistent with earlier reports.

Characterization Of Chitosan By FT-IR And N M R Spectroscopy

FT- IR spectroscopy: To prove that the acid extractable material contains chitosan, its FT-IR spectra were measured in comparison with IR spectrum ofcommercial chitosan from Sigma. All the three isolated pullulan shownsimilar FT- IR spectrum to that of the commercial pullulan. The resultindicated that acid extractable material contains pullulan.

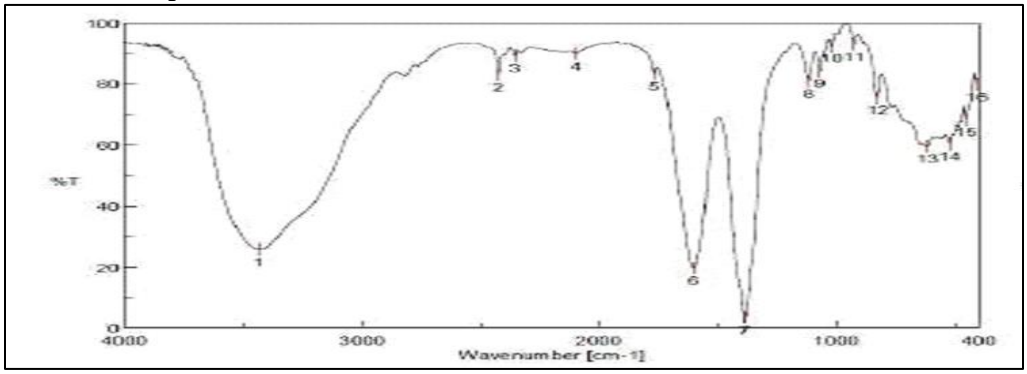

Figure 8: IR Spectrum of Commercial Pullulan

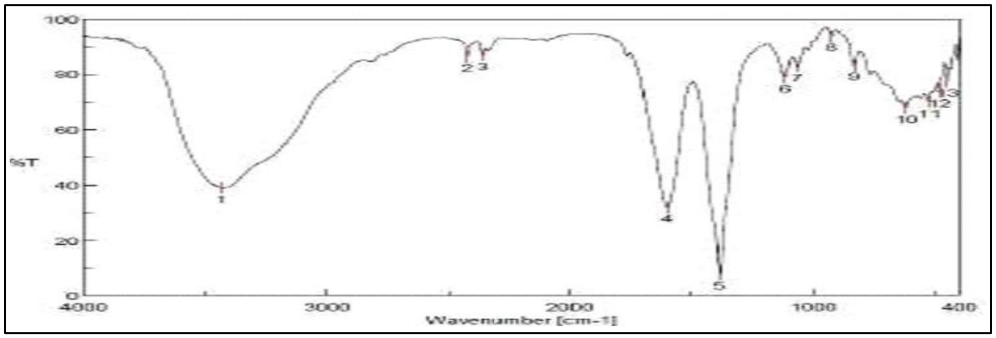

Figure 9: IR Spectrum of Isolated Pullulan from *A.mausonii* NCIM 1226

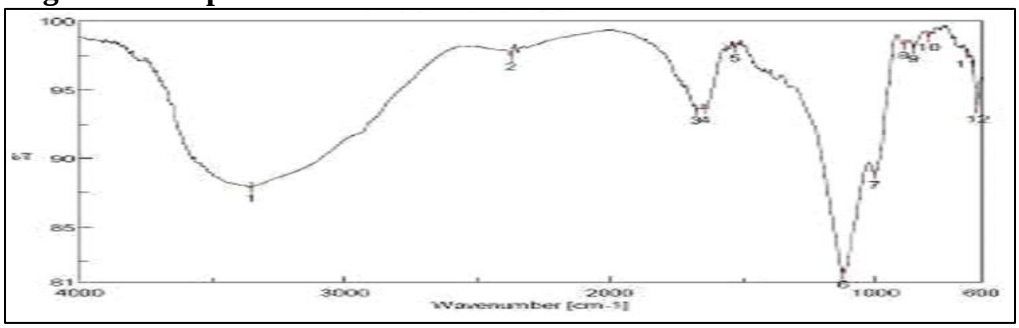

Figure 10: IR Spectrum of Isolated Pullulan from *R. glutinis* NCIM 3168

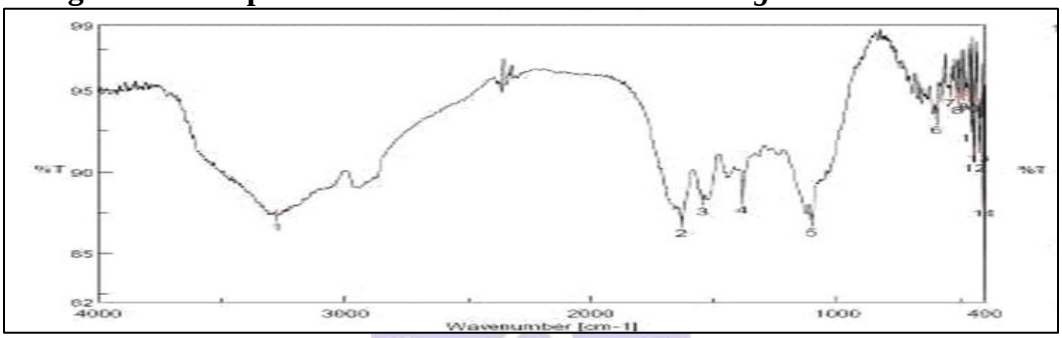

Figure 11: IR Spectrum of Isolated Pullulan from *R.minuta* NCIM 3359

^{1}H NMR Spectroscopy: The ^{1}H NMR spectra was measured in comparison to commercial chitosan and it proved that the isolated fraction contains chitosan. The isolated fraction showed less intense peak at 2.0 ppm (acetyl group) as compared to commercial chitosan confirming **that the isolated chitosan had a higher degree of deacetylation.**

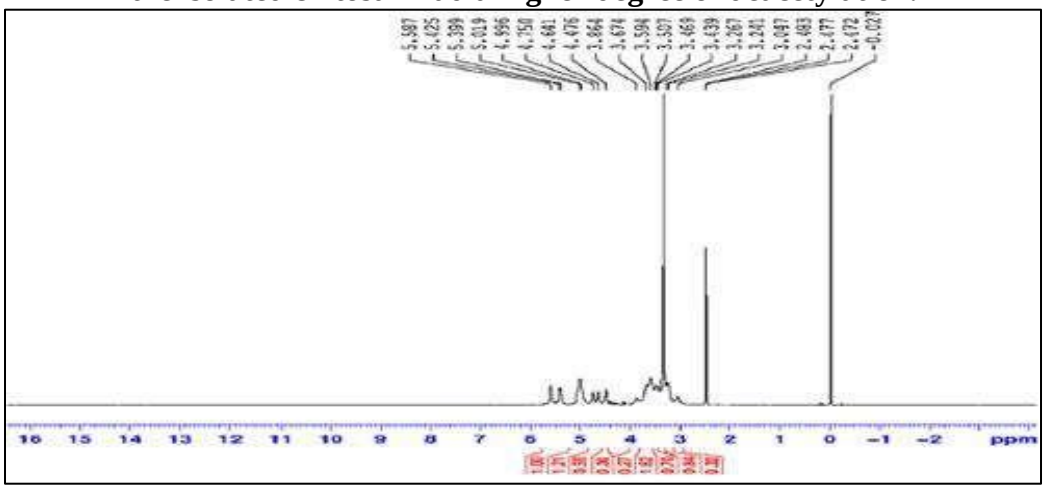

Figure 12: ^{1}HNMR Spectrum of Commercial Pullulan

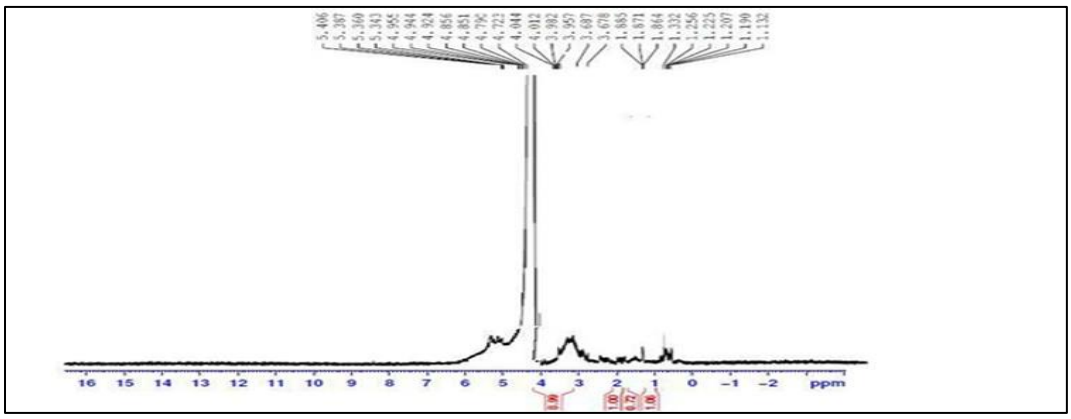

Figure 13: ^{1}HNMR Spectrum of Isolated Pullulan

Conclusion: We have been successful in identifying new microbial strains for the production of pullulan and chitosan (by biotransformation). The strains identified and screened in this research may be used in the polymer industry to produce pullulan with simultaneous production of chitosan economically. These strains possess significant advantages over existing strains in the industry. Factors such as fermentation kinetics, pH, temperature, rpm and inoculum size, effect of carbon & nitrogen sources and concentration of carbon source were found to greatly influence pullulan production and yield. The objective of this research was to identify new strains producing larger amounts of pullulan than existing strains; new strains identified in this research possessed visible industrial advantages for the production of pullulan.

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