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Bioconversion of Various Industrial By- Products and Agricultural Wastes into Pullulan

A. M. Abdel Hafez, Hemmat M. Abdelhady, M. S. Sharaf and T. S. El-Tayeb

Department of Agricultural Microbiology, Fac. of Agric., Ain Shams University, Shoubra El-Kheima, Cairo, Egypt.

Abstract: The production of pullulan by *Aureobasidium pullulans* ATCC 42023 from some available low price industrial by-products and agricultural wastes such as molasses, cellulosic wastes, potato starchy waste, glucose syrup, sweet whey, and corn steep liquor were investigated, using shake flasks as high cell-density-inoculation (HCDI) two-stage batch culture. The maximum pullulan concentration (65.3 gl^{-1}) was obtained after 5 days of fermentation at 28°C using 7% corn steep liquor as the sole nitrogen source in a medium containing 20% sucrose. Under these conditions, the highest values of consumed sugar, conversion coefficient, pullulan yield and productivity were obtained, being 147.9 gl^{-1}, 44.15, 32.65 % and 0.54 gl^{-1} h^{-1}, respectively. The maximum pullulan concentration obtained from other industrial by-products and agricultural wastes were 47.84, 33.21, 22.33, 12.4 and 9.36 gl^{-1} using clarified cane molasses (10% sugars), glucose syrup (5 % sugars), potato starchy waste (3%), enzyme hydrolyzed sweet whey (5% lactose) + 0.05% glutamic + 0.298% KH_2PO_4 and hydrolyzed rice straw (4%) + 1% sucrose, respectively.

Keywords: *A. pullulans*, (HCDI) two-stage batch, pullulan production, industrial by-products, agricultural wastes.

INTRODUCTION

Pullulan is a microbial exopolysaccharide commercially produced by the yeast-like fungus *Aureobasidium pullulans*. The pullulan polysaccharide is a linear homopolysaccharide composed of glucose units. Glucopyranose units are polymerized [linked by α (1→4) glucosidic bonds] into maltotriose units [which are joined by α (1→6) glucosidic bonds][20]. Due to its excellent properties, pullulan is used as a low-calorie ingredient in foods, gelling agent, coating and packaging material for food and drugs, binder for fertilizers and as an oxidation-prevention agent for tablets. Other applications include contact lenses manufacturing, biodegradable foil, plywood, water-solubility enhancer and for enhanced oil recovery[18,11,15].

The use of industrial by-products and agricultural wastes like molasses, potato starchy waste, whey, peat hydrolyzate and brewery wastes effluents as substrates for pullulan production has also been reported by many researchers[4,16,2]. Utilization of these substrates would seem to be ecologically sound and economically advantageous as they have low or even negative costs[11].

West & Strohfus[21] studied Pullulan production by *Aureobasidium pullulans* RP-1 (ATCC 201253) using 2.5-10% (w/v) corn syrup as carbon source and corn steep solids (0.06 or 0.2% w/v) as a nitrogen source. With 2.5% corn syrup, produced pullulan was greater when corn steep liquor was replaced by ammonium sulfate as the N source. With 5, 7.5 or 10 % corn syrup in the medium, less pullulan was produced when the N source was corn steep liquor instead of ammonium sulfate where, supplementation of the medium with yeast extract slightly influenced the amount of pullulan produced. At all the corn syrup concentrations tested, the medium containing ammonium sulfate gave higher fungal growth than medium containing corn steep liquor after 7 days fermentation period. Barnett *et al.,*[2] found that enzymatic hydrolysis of potato starchy waste yielded a substrate suitable for *A. pullulans* growth and exopolysaccharide production. Hydrolysates produced by α-amylase alone, gave the lowest yield of exopolysaccharide. Continued hydrolysis with pullulanase and amyloglucosidase gave higher yields of pullulan, but prolonged hydrolysis did not improve the yield further. Maltose-rich hydrolyzates, generated with β-amylase and pullulanase, yielded over twice as much pullulan as the corresponding glucose-rich syrups generated with amyloglucosidase and pullulanase. The maximum pullulan concentration achieved was 27 gl^{-1} with 10 % (w/v) potato starchy waste.

Corresponding Author: A. M. Abdel Hafez, Department of Agricultural Microbiology, Fac. of Agric., Ain Shams University, Shoubra El-Kheima, Cairo, Egypt.

Roukas[17] studied the production of pullulan from deproteinized whey by *A. pullulans* P56 using an adaptation technique and a mixed culture system. The adaptation of *A. pullulans* and the mixed cultures of *A. pullulans* and/or *Lactobacillus brevis* X20, *Debaryomyces hansenii* 194 and *Aspergillus niger* did not increase the production of polysaccharide. Enzymatic hydrolysis of lactose in deproteinized whey gave a higher polysaccharide concentration and yield than acidic hydrolyzed lactose. Maximum polysaccharide concentration (11.0 gl^{-1}), biomass dry weight (10.5 gl^{-1}) and polysaccharide yield (47.2 %) were achieved using enzyme-hydrolyzed whey containing 25 gl^{-1} lactose and supplemented with 0.5 % K_2HPO_4, 1 % L-glutamic acid, 2.5 % olive oil, and 0.5 % Tween 80. Lazaridou *et al.,*[14] studied the production of pullulan from beet molasses by a pigment–free strain of *Aureobasidium pullulans* on shake-flask culture. Combined pretreatment of molasses with sulfuric acid and activated carbon to remove potential fermentation inhibitors present in molasses resulted in a maximum pullulan concentration of 24 gl^{-1}, biomass dry weight of 14 gl^{-1}, a pullulan yield of 52.5 %, and a sugar utilization of 92 % with optimum fermentation conditions (initial sugar concentration of 50 gl^{-1} and initial pH of 7.0). The addition of other nutrients as carbon and nitrogen supplements (olive oil, ammonium sulfate, yeast extract) did not further improve the production of pullulan. GÖksungur *et al.,*[9] obtained a maximum pullulan concentration of 16.7gl^{-1} in modified medium whereas 16.9 gl^{-1} of pullulan were obtained in molasses medium containing 50 gl^{-1} of initial sugar at pH 7.5.

In this study, molasses, cellulosic wastes, potato starchy waste, glucose syrup, sweet whey, and corn steep liquor as industrial by-products and agricultural wastes, pretreated with different techniques were compared (alone or with additional nutrition) with a modified synthetic medium for pullulan production by *A. pullulans* ATCC 42023 using shake flasks as a HCDI two-stage batch cultures to reduce production costs. Effect of different concentrations of molasses, potato starchy waste, glucose syrup and CSL on pullulan production was studied. Combinations between selected wastes as complementary substrates for pullulan production were also studied.

MATERIALS AND METHODS

Fungal Strain: *Aureobasidium pullulans* ATCC 42023 was obtained from American type culture collection, subcultured on malt agar slants at 30°C and maintained at 4°C.

Media: Malt agar medium[1] was used for propagation and preservation of aureobasidium culture. Modified Reeslev & Jensen medium[6], was used as basal medium for pullulan production after replacement of its carbon or nitrogen source with a certain industrial by-product or agricultural waste.

Preparation of Standard Inoculum: Standard inoculum was prepared by transferring a loop of the tested culture into 250 ml conical flasks containing 50 ml of modified Reeslev & Jensen medium. The inoculated flask was incubated on a rotary shaker at 210 rpm for 48 h at 30°C**.** The content of this flask was used as a standard inoculum (1 ml contained 6.0–7.0 x 10^5 viable cells). The inoculum was prepared by centrifugation at 12000 x g for 15 min, and then cells were washed twice with sterile distilled water and harvested to inoculate productive medium as a HCDI two-stage batch experiments as the method described by Shabtai & Mukmenev[19].

Fermentation Process and Cultural Conditions: Fermentation was carried out in 250 ml Erlenmeyer flasks, each containing 100 ml sterile productive medium. The inoculated flasks were then incubated at 28°C using a rotary shaker at 210 rpm. Samples (10 ml) were taken from the growing culture after 5 days of fermentation under aseptic conditions. The biomass was separated by centrifugation at 4000 X g for 10 min and the sediment was washed twice with distilled water and then dried at 70°C for constant weight. Pullulan concentration and pH were also determined in the supernatant.

Industrial By-products and Agricultural Wastes: Some industrial by-products and agricultural wastes were used for pullulan production. These materials were used as nitrogen source like CSL or carbon source adjusted from 5 to 20% total sugars alone or in combination with other constituents of modified Reeslev & Jensen medium. In addition, effect of different concentrations of molasses, potato starchy waste, glucose syrup and CSL on pullulan production was studied. These materials were obtained from different sources and were varied in their content of total sugars and nitrogen as shown in Table (1):

Pretreatment of Industrial By-products and Agricultural Wastes: Black strap cane molasses was diluted by addition of water in a ratio of 1: 1, acidified to pH 4.0, heated in a water bath at 100°C for 1 h, and then kept overnight to precipitate the undesirable metal salts.

Table 1: Chemical analyses of industrial by-products and agricultural wastes used for pullulan production.

Industrial by-products and agricultural wastes	Source	Total sugars (%)	Total nitrogen(%)
1-Black strap cane molasses	Sugar refinery factory at El - Hawamdia	48.3	0.102
2- High test cane molasses	Local market, Cairo	71.4	0.17
3- Wheat and rice straw	Farms of wheat and rice in Al Behera	-	-
4- Sawdust	Wood workshops in Cairo	-	-
5- Potato starchy waste	Farm frits factory at 10^{th} Ramadan city	40	0.15
6- Glucose syrup	Glucose and Starch Co. Torrah, Cairo	42	0.28
7- Sweet whey	Egypt Dairy Products Co. El – Amereia, Cairo	5	0.8
8- Corn steep liquor	Glucose and Starch Co. Torrah, Cairo	0.5	4.64

Cellulosic wastes (sawdust, wheat and rice straw) were exposed to four different treatments in order to convert cellulose content into more available sugars, these treatments were carried out according to Deshpande *et al.,*[5] as follows:

- Treatment (A): 5 % cellulosic waste was hydrolyzed with 0.25 M H_2SO_4, sterilized at 121°C, 20 min. and then neutralized with NaOH to pH 4.5 – 5.0.
- Treatment (B): 5 % cellulosic waste was hydrolyzed with 0.7 % H_2SO_4, sterilized at 2 p.s.i., 60 min. and then neutralized with NaOH to pH 7.0.
- Treatment (C): 5 % cellulosic waste was hydrolyzed with 0.5 M H_2SO_4, sterilized at 121°C, 40 min. and then neutralized with NaOH to pH 7.0.
- Treatment (D): 4 % cellulosic waste was hydrolyzed with 0.5 M H_2SO_4, sterilized at 121°C, 40 min. and then neutralized with NaOH to pH 7.0. 1 % sucrose was added as carbon source in this treatment.

Sweet whey was hydrolyzed by b - galactosidase or HCl in order to convert lactose into glucose and galactose, where *A. pullulans* strains cannot use lactose as an efficient carbon source. Enzyme hydrolization was carried out according to the method of[17]. At first protein precipitation was induced by heating the whey at 90°C for 20 min. Precipitated proteins were removed by centrifugation at 5000 × g for 15 min. One liter of the supernatant (pH 6.5) was hydrolyzed with 2.0 ml of β-galactosidase at 40°C for 5 h in a rotary shaking/incubator at 120 rpm. After hydrolysis, the solution was sterilized at 121°C for 20 min. In case of acidic hydrolysis, the pH of the supernatant was adjusted to 1.5 with HCl and the medium was heated at 121°C for 30 min. After cooling the medium, pH was adjusted to 7.0 with 10 N NaOH. Potato starchy waste collected from potato factory was dried by exposing to the direct sunlight for 48 h to get rid of its water content. All by-products or wastes were stored in the refrigerator at 5 –7°C until used.

Pullulan Determination: Pullulan was precipitated in the culture supernatant with 2 volumes of ethanol 99%, at 4°C for 1 h. The precipitate was centrifuged at 4000 x g for 10 min followed by drying at 80°C overnight and was then weighed[9].

Chemical Determinations: Total residual sugars were determined in the fermented liquor according to the method described by Flood & Priestly[7]. Total nitrogen was determined using Kjeldahel method as described by Jackson[12].

Parameters Related to Pullulan Production: Pullulan yield coefficient relative to biomass ($Y_{p/x}$), conversion coefficient (%) pullulan yield (%) and productivity (P) were calculated according to Gamal *et al.,*[8].

RESULTS AND DISCUSSIONS

Effect of Some Industrial By-products and Agricultural Wastes on Pullulan Production by *A. pullulans* Atcc 42023 Using Shake Flasks as HCDI Two-stage Batch Culture:

Molasses: Clarified cane molasses and high-test cane molasses were used for pullulan production by *A. pullulans* ATCC 42023. Data recorded in Table (2) indicates that high-test cane molasses treatment enhanced both biomass formation and pullulan production (18.9 and 46.5 gl^{-1}) than clarified cane molasses treatment. The former treatment increased cell dry weight and pullulan concentration by 1.1 and 1.04 fold, respectively comparing with modified

Table 2: Growth of *A. pullulans* ATCC 42023 and pullulan production on cane molasses (20% sugars) treatments after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Treatments	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$(gg^{-1})	Pullulanyield (%)	Final pH
Clarified cane molasses	17.11	44	2.57	22	2.7
Clarified cane molasses + (modified Reeslev & Jensen medium – sucrose)	15.2	31.92	2.1	15.96	3.9
Clarified cane molasses + KH_2PO_4	18.1	41.04	2.27	20.52	4.1
High test cane molasses	18.9	46.5	2.46	23.25	2.2
High test cane molasses + (modified Reeslev & Jensen medium – sucrose)	17.9	36.9	2.06	18.45	3.5
High test cane molasses + KH_2PO_4	14.4	34.6	2.39	17.3	4.0
Modified Reeslev & Jensen medium (control)	16.65	44.61	2.68	22.3	2.5

Table 3: Pullulan production by *A. pullulans* ATCC 42023 as influenced by different sugar concentrations of clarified cane molasses after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Sugars (%) of clarified cane molasses	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$(gg^{-1})	Pullulanyield (%)	Final pH
5	13.1	20.36	1.55	40.72	2.6
10	14.2	47.84	3.37	47.84	2.2
15	17.3	45.3	2.62	30.02	3.2
20 (control)	18.1	44.4	2.45	22.2	4.3
25	14.74	15.3	1.03	10.4	5.6

Table 4: Pullulan production by *A. pullulans* ATCC 42023 as influenced by different acid hydrolysis of milled wheat straw, rice straw or sawdust (as carbon sources) in modified Reeslev & Jensen medium after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Cellulosic waste	Treatments	Pullulan concentration (gl^{-1})	Pullulan yield (%)	Final pH
Wheat straw	A	2.2	4.4	4.5
	B	3.2	6.4	5.2
	C	3.51	7.02	5.7
	D	8.88	17.76	4.2
Rice straw	A	2.28	4.56	5.6
	B	3.61	7.22	4.2
	C	4.5	9	4.7
	D	9.36	18.7	4.0
Sawdust	A	1.24	2.48	4.0
	B	2.3	4.6	5.8
	C	2.34	4.68	6.1
	D	3.2	6.4	5.6
Modified Reeslev & Jensen medium (control)	_	20.01	40.2	2.5

A, B and C: 5 % waste was hydrolyzed by H_2SO_4, at different concentrations.
D: Hydrolyses of 4 % waste + 1 % sucrose.

Reeslev & Jensen medium (control). Adding minerals of modified Reeslev & Jensen medium, or KH_2PO_4 to cane molasses treatments observed a bad effect on pullulan production. The highest figures of pullulan yield (23.25 %) and pullulan yield coefficient relative to biomass (2.57) were achieved using high test cane molasses and clarified cane molasses. The lowest final pH (2.2) was recorded when using high test cane molasses. Also, it could be noticed that using clarified cane molasses gave approximately the same results obtained from modified Reeslev & Jensen medium besides its cheaper price than high-test cane molasses, it will be chosen for further experimentation. Therefore, detecting the optimum concentration of dissolved sugars in clarified cane molasses for maximum pullulan production was recorded in Table (3). Data clearly show that using 10% sugars of clarified cane molasses was the most appropriate concentration for high pullulan production, being 47.84 gl^{-1}. At this concentration the highest figures of pullulan yield coefficient relative to biomass and pullulan yield (3.37 gg^{-1} and 47.84%) were obtained, increasing pullulan yield by 2.15 fold than the control concentration (20% sugars). The lowest final pH (2.2) was recorded at 10% sugars.

Depending on these results, it could be stated that clarified cane molasses (10% sugars) was a suitable by-product for HCDI two-stage batch pullulan production. In this respect, Lazaridou *et al.,*[14] achieved a maximum pullulan concentration of 24 gl^{-1} using beet molasses medium (5% sugars) after 192 h. Also, GÖksungur *et al.,*[9] obtained 16.9 gl^{-1} pullulan in a molasses medium containing 50 gl^{-1} sugars at pH 7.5.

Cellulosic Wastes: Cellulosic wastes are produced with huge quantities allover the country. The discarding of these tons of wastes always considered a terrible problem. This is due to the expensive cost of transferring these wastes and the pollution from discarding them by burning. In the trials for using these wastes as substrates for pullulan fermentation, data given in Table (4) show the pullulan production after five days incubation period on different acid hydrolyzates of some cellulosic wastes, such as wheat straw, rice straw and sawdust. It could be noticed that acid hydrolyses of 4 % cellulosic waste plus 1 % sucrose (treatment D) gave the highest pullulan production among all other treatments, in this treatment, the highest figures of pullulan yield and concentration were obtained on acid hydrolyzate of rice straw being 18.7 % and 9.36 gl^{-1} followed on acid hydrolyses of wheat straw being 17.76 % and 8.88 gl^{-1}, respectively. The failure of other treatments of rice straw to achieve the same production could be attributed to the absence of sucrose. Modified Reeslev & Jensen medium was still superior for pullulan production. Therefore, the cellulosic wastes were not suitable substrates for high pullulan production.

Table 5: Growth of *A. pullulans* ATCC 42023 and pullulan production on 5% potato starchy waste treatments after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Treatments	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$(gg^{-1})	Pullulan yield (%)	Final pH
Potato starchy waste (5%)	8.33	5.44	0.65	10.88	4.1
Potato starchy waste (5%) + (modified Reeslev & Jensen medium – sucrose)	9.31	22.5	2.42	45	3.2
Potato starchy waste (4.5%) + (modified Reeslev & Jensen medium with sucrose 0.5%)	9.2	11.52	1.25	23.04	3.8
Modified Reeslev & Jensen medium (control)*	16.2	20.21	1.25	40.42	2.5

*: Modified Reeslev & Jensen medium (5% sucrose).

Table 6: Pullulan production by *A. pullulans* ATCC 42023 as influenced by different concentrations of potato starchy waste as carbon source in modified Reeslev & Jensen medium after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Potato starchy waste concentrations (%)	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$(gg^{-1})	Pullulan yield (%)	Final pH
2	5.7	13.44	2.36	67.2	4.3
3	6.66	22.33	3.35	74.4	3.5
5 (control)	9.88	22.25	2.25	44.5	4.1
10	4.5	7.1	1.58	7.1	5.2
15	3.56	5.34	1.5	3.56	6.3
20	2.71	2.12	0.78	1.06	6.6

Acid straw hydrolyzed by 0.25 M sulfuric acid was used by Han *et al.,*[10] to obtain 5.6 gl^{-1} pullulan concentration. Leathers[15] found *Aureobasidium* sp., color variant strain NRRL Y – 12974, grew on basal medium containing either corn fiber or corn condensed distiller's solubles (CCDS) as a carbon source and produced pullulan with little melanin contamination.

Potato Starchy Waste: Potato starchy waste is an industrial product of the semi–fried potatoes factories. This waste has been shown to be a suitable feedstock for industrial fermentations and is comparable to traditional substrates such as molasses and syrups in many cases. Disposal of potato starchy waste is a problem due to the high biological oxygen demand when it is placed in the local sewage system. It was found to contain 40% total carbon and 0.83% total nitrogen. Therefore, it was used at the concentration of 5% as the sole carbon source in different treatments included only potato starchy waste, or added to modified Reeslev & Jensen medium at 5% or 4.5% plus 0.5% sucrose.

Data recorded in Table (5) show that the lowest pullulan concentration (5.44 gl^{-1}) was produced by *A. pullulans* ATCC 42023 in only 5% potato starchy waste, this might be due to the deficiency of nitrogen or mineral content of potato starchy waste. In contrast, a remarkable increase in pullulan content was detected when potato starchy waste was added to modified Reeslev & Jensen medium as the sole carbon source, and this gave the highest figures of pullulan concentration (22.5 gl^{-1}), pullulan yield coefficient relative to biomass (2.42 gg^{-1}) and pullulan yield (45 %). This indicated a stimulating effect of potato starchy waste on pullulan production but not on *A. pullulans* ATCC 42023 growth. Comparing pullulan concentration of this treatment with that produced on modified Reeslev & Jensen medium (control), it could be noticed that it increased about 11.3%.

No remarkable increase in pullulan production was obtained at this treatment with increasing or decreasing the concentration of potato starchy waste than 5% as shown in Table (6). These results are not in line with those of Barnett *et al.*,[2] they found that enzymatic hydrolysis of potato starchy waste was essential for pullulan production. The maximum pullulan concentration achieved was 27 gl^{-1} with (10%) w/v potato starchy waste.

Glucose Syrup: Glucose syrup is a refined, concentrated aqueous solution of D (+) – glucose, maltose and other sugars of D – glucose obtained by the controlled partial hydrolysis of edible starch. Glucose syrup has unique sugar content, that it contains monosaccharides, disaccharides, trisaccharides, tetrasaccharides, pentasaccharides, hexasaccharides and higher sugars[13].

Data presented in Table (7) show that cell dry weight, pullulan concentration and pullulan yield of all different glucose syrup treatments were lower than

Table 7: Growth of *A. pullulans* ATCC 42023 and pullulan production on different glucose syrup treatments after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Treatments	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$(gg^{-1})	Pullulanyield (%)	FinalpH
Glucose syrup (20% sugars)	4.32	8.8	2.03	4.4	3.5
Glucose syrup (20% sugars) + (modified Reeslev & Jensen medium – sucrose)	4.98	13.5	2.71	6.75	2.2
Glucose syrup (20% sugars) + 0.05 % glutamic acid + 0.298 % KH_2PO_4	4.12	7.2	1.75	3.6	2.4
Modified Reeslev & Jensen medium (20% sucrose) (control)	16.88	45.44	2.7	22.72	2.8

Table 8: Pullulan production by *A. pullulans* ATCC 42023 as influenced by different glucose syrup sugars concentrations as carbon source in modified Reeslev & Jensen medium after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Glucose syrup concentrations added to modified Reeslev & Jensen medium - sucrose (% sugars)	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$(gg^{-1})	Pullulanyield (%)	Final pH
3	11.7	18.1	1.55	60.3	4.4
5	13.2	33.21	2.52	66.4	3.3
10	9.21	22.8	2.48	22.8	4.6
15	6.4	17.6	2.75	11.7	5.2
20 (control)	4.45	13.33	3.0	6.7	6.4

Table 9: Growth of *A. pullulans* ATCC 42023 and pullulan production on different treatments of sweet whey (5% lactose) after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Treatments	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$(gg^{-1})	Pullulanyield (%)	FinalpH
Sweet whey (5% lactose)	2.41	5.5	2.28	11	3.5
Sweet whey (5% lactose)+ (modified Reeslev & Jensen medium – sucrose)	2.66	8.65	3.26	17.3	3.2
Acid hydrolyzed sweet whey (5% lactose)	3.12	7.6	2.44	15.2	3.8
Enzyme hydrolyzed sweet whey (5% lactose)	3.5	10.16	2.9	20.32	2.6
Enzyme hydrolyzed sweet whey (5% lactose) + 0.05 % glutamic + 0.298 % KH_2PO_4	2.78	12.4	4.46	24.8	2.3
Modified Reeslev & Jensen medium * (control)	15.6	20.6	1.32	41.2	2.6

* Modified Reeslev & Jensen medium (5% sucrose).

Table 10: Growth of *A. pullulans* ATCC 42023 and pullulan production on corn steep liquor treatments after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Treatments of CSL	Cell dry weight (gl^{-1})	Pullulan concentration (gl^{-1})	$Y_{p/x}$(gg^{-1})	Pullulanyield (%)	FinalpH
Clarified cane molasses (10 % sugars) + 10 % CSL	7.48	22.6	3.02	22.6	3.1
3 % Potato starchy waste + (modified Reeslev & Jensen medium – sucrose) + 10 % CSL	4.89	24.1	4.92	80.3	4.1
Glucose syrup (5% sugars) + modified Reeslev & Jensen medium – sucrose + 10 % CSL	12.5	40.4	3.23	80.8	2.4
Enzyme hydrolyzed sweet whey (5% lactose) + 10 % CSL	10.2	16.04	1.57	32.08	3.6
[Modified Reeslev & Jensen medium (20%) sucrose – glutamic] + 10 % CSL	18.8	62.64	3.33	31.32	2.2
Modified Reeslev & Jensen medium (20%) sucrose + 10 % CSL	17.45	50.3	2.88	25.15	2.5
(20 %sucrose) + (10% CSL)	8.2	29.1	3.55	14.55	2.2
Modified Reeslev & Jensen medium (20%) sucrose	16.2	45.84	2.83	22.92	2.3

that obtained from modified Reeslev & Jensen medium, whereas, supplementation of the latter medium with glucose syrup (20% sugars) gave a high pullulan concentration (13.5 gl^{-1}) and recorded the same pullulan yield coefficient relative to biomass of modified Reeslev & Jensen medium (2.71 gg^{-1}).

From data given in Table (8) it could be noticed that decreasing the sugars concentration of glucose syrup in modified Reeslev & Jensen medium than 20% increased the cell dry weight and pullulan to reach the maximum at 5% sugars being 13.2 and 33.21 gl^{-1}, respectively. The corresponding figures of pullulan yield coefficient relative to biomass and pullulan yield were 2.52 gg^{-1} and 66.4%, respectively. This might be due to the presence of some significant inhibitory materials at high concentrations of glucose syrup.

Generally, it could be stated that using glucose syrup (5% sugars) as a sole carbon source in modified Reeslev & Jensen medium increased the pullulan yield about 43.7% than that recorded from modified Reeslev & Jensen medium (20% sucrose).

Sweet Whey: Sweet whey is a nutrient–rich dairy by-product which contains 5 % lactose, 0.8 % to 1 % proteins, minerals, and some small organic molecules[3]. Data given in Table (9) reveal that the highest *A. pullulans* ATCC 42024 growth (3.5 gl^{-1}) was obtained on enzyme hydrolyzed sweet whey (5% lactose) as carbon source. Whereas, the highest pullulan concentration (12.4 gl^{-1}) was observed on enzyme

Table 11: Pullulan production by *A. pullulans* ATCC 42023 as influenced by different concentrations of corn steep liquor in modified Reeslev & Jensen medium after 5 days of incubation at 28°C using shake flasks as a HCDI two-stage batch culture.

Modified Reeslev Jensen medium content CSL(%)*	Cell dry weight (gl^{-1})	Pullulan conc (gl^{-1})	$Y_{p/x}$ (gg^{-1})	Consumed sugar (gl^{-1})	Residual sugars (gl^{-1})	Conversion coefficient (%)	Pullulan yield (%)	Porductivity (gl^{-1} h^{-1})	Final pH
1	7.1	25.7	3.62	61.2	138.8	41.9	12.85	0.21	3.1
4	13.8	52.15	3.78	120.5	79.5	43.2	26.08	0.43	2.4
7	19.2	65.3	3.4	147.9	52.1	44.15	32.65	0.54	2.2
10 (control)	18.2	61.1	3.36	143	57	42.7	30.55	0.5	2.3
13	17.6	42.6	2.42	98.8	101.2	43.1	21.3	0.35	2.2
16	15.3	30.12	1.97	78.1	121.9	38.6	15.06	0.251	2.2
Modified Reeslev & Jensen medium (20% sucrose)	14.5	45.3	3.12	122	78	37.1	22.65	0.38	2.3

* Modified Reeslev & Jensen medium (20% sucrose) – glutamic acid + CSL (%).

hydrolyzed whey supplemented with 0.05% glutamic acid and 0.298 % KH_2PO_4, after five days incubationperiod, which was about 39.8% lower than the control. This might be due to the influence of glutamic acid on enhancing pullulan production, resulting the highest values of pullulan yield and pullulan yield coefficient relative to biomass of 24.8 % and 4.46 gg^{-1}, respectively. Also, it could be noticed that the lowest pH value being 2.3 was recorded in this treatment at the end of fermentation period, and the high pullulan production was accompanied with a decrease in culture pH. These results indicated that sweet whey as a substrate for pullulan production requires additional trials to enhance its production and reduce the hydrolysis costs.

These results are in line with those obtained by Roukas[17] who stated that high pullulan production was obtained during the fermentation of enzyme hydrolyzed deproteinized sweet whey (11.0 ± 0.5 gl^{-1}). Enzymatic hydrolysis of lactose in deproteinized sweet whey gave higher pullulan concentration and pullulan yield than acidic hydrolyzed lactose.

Corn Steep Liquor: Corn steep liquor (CSL) is a by-product produced during the manufacture of starch and other corn products. CSL (10%) was added to sucrose solution (20%), complete modified Reeslev & Jensen medium, modified Reeslev & Jensen medium free of glutamic acid and the best treatments of industrial wastes in order to select the best treatment for pullulan production. Data recorded in Table (10) show that the addition of CSL to 20% sucrose solution gave the lowest pullulan yield (14.55%). This may be due to the deficiency of nitrogen and mineral sources. Also, all industrial wastes treatments containing 10% CSL gave lower pullulan concentration than modified Reeslev & Jensen medium. Comparing the results of pullulan production of these treatments with those obtained on industrial wastes treatments without CSL addition, it is interesting to notice that pullulan concentration increased with 8, 21.7 and 58% on 3% potato starchy waste, glucose syrup (5% sugars) and enzyme hydrolyzed sweet whey (5% sugar) and decreased with 53% on clarified cane molasses (10% sugars), as shown in Tables (6, 8, 9 and 3, respectively).

In contrast, a remarkable increase in pullulan concentration was detected when 10 % CSL was added to complete modified Reeslev & Jensen medium or modified Reeslev & Jensen medium without glutamic acid, being 50.3 and 62.64 gl^{-1}, which increased by 1.1 and 1.37 fold, respectively than modified Reeslev & Jensen medium without CSL. Moreover, pullulan concentration was increased to a maximum (65.3 gl^{-1}) when CSL concentration decreased from 10% to 7% as shown in Table (11). Also, the highest figures of consumed sugar, conversion coefficient, pullulan yield and productivity were obtained at 7 % CSL being 147.9 gl^{-1}, 44.15, 32.65 % and 0.54 $gl^{-1}h^{-1}$, respectively. At the end of incubation period (5 days), the final pH was decreased from 3.1 to 2.2 with increasing of CSL concentrations from 1.0 to 7.0 %. This may be due to the release of some organic acids from CSL amino acids after the nitrogen consumption for biomass formation (ranged from 7.1 to 19.29 gl^{-1}). Therefore, it could be stated that using 7 % CSL as a sole nitrogen source in modified Reeslev & Jensen medium (20% sucrose) was the most efficient among all industrial by-products and agricultural wastes propagations to achieve the highest pullulan production by *A. pullulans* ATCC 42024 as seen in Fig. (1).

West & Strohfus[21] added corn steep liquor (0.06 or 0.2 % w/v) as nitrogen source for pullulan production by *Aureobasidium pullulans* RP-1 (ATCC 201253) in media containing 2.5 - 10 % (w/v) corn

syrup as carbon source. They found that with 2.5 % corn syrup as carbon source, pullulan production was greater when corn steep liquor replaced ammonium sulfate as the N source, regardless of supplementation with 0.04 % yeast extract. With 5, 7.5 or 10% corn syrup in the medium, less polysaccharide was produced when the N source was corn steep liquor instead of ammonium sulfate where, supplementation of the medium with yeast extract slightly influenced the amount of pullulan produced.

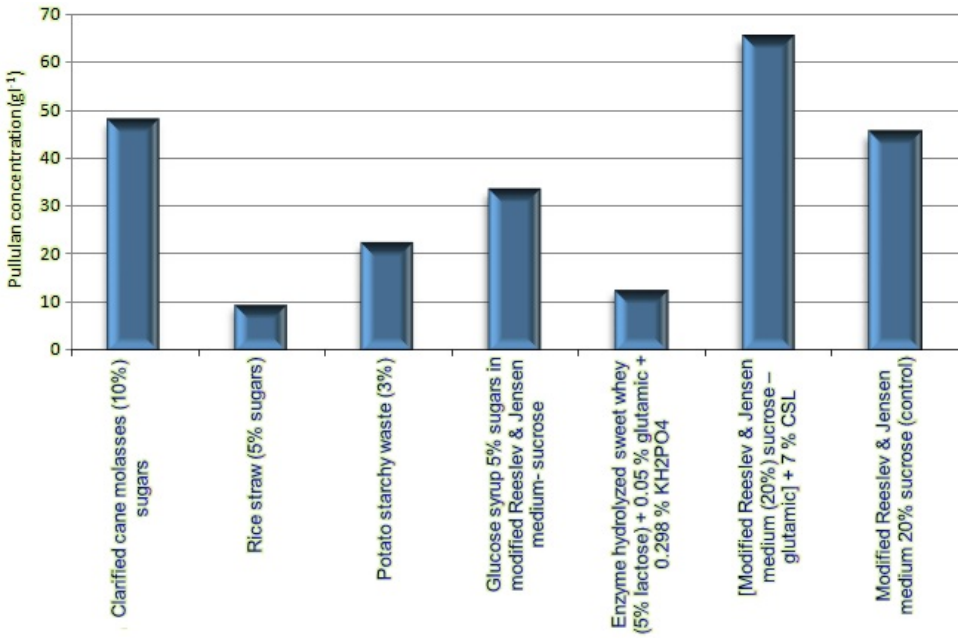

Fig. 1: Effect of some industrial by-products and agricultural wastes on pullulan production by A. *pullulans* ATCC 42023 after 5 days of incubation at 28°C using shake flasks as HCDI two-stage batch culture.

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