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Yeast Growth Potential and COD Reduction in Waste Water from Ergot Alkaloid Production

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Summary

The screening of yeast cultures for their growth and chemical oxygen demand (COD) reduction capability in waste water from ergot alkaloid production was evaluated. Efficient COD reduction in filtered waste water was the main criterion for the screening of 27 previously selected yeast strains from the Culture Collection of Industrial Microorganisms (ZIM), Ljubljana, Slovenia. Sixteen 16 isolated yeast strains originating from the waste water were also included. The results showed that 3 yeasts from the ZIM Culture Collection reduced COD by over 60 % and 10 out of 16 isolated yeast strains reduced 68 to 74 % of the initial COD value within 72 hours. Final screening among yeast cultures was performed in a 2 L airlift bioreactor. It was found that 10 yeast isolates, identified as Pichia anomala strains could be used for reducing the COD load of the waste water from ergot alkaloid production. One of the isolated yeasts, ZIM 419, was further cultivated in a 3.5 L stirred bioreactor and 81 % reduction of the initial COD was achieved within 37 hours.

Key words: COD reduction, ergot alkaloid, waste water, yeast

Introduction

Effluent limitations and the costs of discharging waste water into waste water treatment plants are forcing industry into its pre-treatment. Waste waters from the pharmaceutical industry have a high COD load and there is also the possibility of residual substances being produced, which could have an effect on the microbial community in a waste water treatment plant. When the microbial community is affected this can lead to the pollution of the natural environment because waste water is drained off without efficient treatment.

Conventionally, the waste water is treated by removing the oxygen-consuming organic matter in the treatment process, including removal of the suspended solids by sedimentation in the first step and removal of the soluble organic matter by biological oxidation in a biological or secondary treatment. The biological treatments have an advantage in comparison with chemical treatments because of the biocatalytical capability of microorganisms in terms of lower energy costs *(1)*. Waste waters released from pharmaceutical industry are heterogeneous and complex, some of them rather toxic, especially when the drugs are produced by chemical synthesis *(2)*. Pharmaceutical waste waters can undergo aerobic or anaerobic biological treatments combined with physical or chemical methods depending on their composition and toxicity *(2–5)*. However, waste waters from fermentation processes, even in pharmaceutical industry, show similarities with other polysaccharide-containing waste waters, because they have high organic load with easily biodegradable compounds *(4,7)*.

The objective of the present study was to evaluate the capability of various yeasts to grow in the waste water generated from ergot alkaloid production in Lek Pharmaceutical & Chemical Company Ljubljana. Ergot alkaloids are produced in a fermentation process by the fungus *Claviceps purpurea*, and the exact composition of the media in terms of the remaining compounds is not specified. Waste waters remaining after ergot alkaloid production and downstream processing are rich in sugars, nitrogen compounds, metabolic products of the fungus *Claviceps purpurea* and organic solvents used for ergot alkaloid extraction from the media.

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Materials and Methods

Yeast strains

Sixty yeast strains were obtained from the Culture Collection of Industrial Microorganisms (ZIM) from the Biotechnical Faculty, Ljubljana, Slovenia and sixteen yeast strains were isolated from a waste water collecting tank of Lek Pharmaceutical & Chemical Company. Tables 2 and 3 list the strains selected and used in this study. The isolated yeast strains ZIM 85 (NCAIM P Y 1247) and ZIM 419 (NCAIM P Y 1248) have also been deposited in the National Collection of Agricultural and Industrial Microorganisms, NCAIM, Budapest.

Waste water

The waste water was collected from Lek Pharmaceutical & Chemical Company Ljubljana, Slovenia, which produces ergot alkaloids. 100 L of waste water were collected without any treatment in 25 L plastic containers. A further 200 L of waste water were treated with flocculent Primafloc A-10 (Rohm and Haas Company) and filtered. Both the waste water and its filtrate (Table 1) were kept in a deep-freezer at –18 °C.

Inoculum preparation

The yeasts from the refrigerated stock culture were transferred to malt extract agar plates containing (g/L): malt extract 30, yeast extract 4, glucose 4, agar 20 and incubated at 28 °C for 48–72 hours. For the shake culture experiment the yeast was transferred from the plate to a test tube containing 5 mL of sterile filtered waste water to a turbidity of 0.2 determined spectrophotometrically at 650 nm.

For the experiments performed in airlift and stirred bioreactors the inoculum was prepared as follows: a loopful of yeast was transferred to a 200 mL Erlenmeyer flask containing 50 mL of sterile filtered waste water and the flask was shaken at 28 °C for 15 hours. For the airlift bioreactor 15 mL of this preculture was inoculated in a 500 mL Erlenmeyer flask with 60 mL of sterile filtered waste water. In the case of the stirred bioreactor the whole volume of preculture was inoculated in a 1 000 mL Erlenmeyer flask with 210 mL of sterile filtered waste water. The inoculum was shaken at 28 °C for 18–20 hours.

Shake flask experiments

Experiments were carried out using 47.5 mL of filtered sterile waste water in 150 mL Erlenmeyer flasks inoculated with 2.5 mL of yeast suspension. The flasks were placed on a rotary shaker at 200 r.p.m. for 72 hours at 28 °C. A control sample containing only the filtered sterile waste water was included in the experiment.

Airlift bioreactor experiments

The bioprocess was performed in batch mode in a 2 L bioreactor at 28 °C and aeration set up to 1.5 L air/L waste water/min. The bioreactor was filled with 1.44 L of filtered sterile waste water and 5 % of yeast inoculum was added. Samples were taken every 12 hours. The control sample containing the filtered sterile waste water was subjected to the same experimental conditions.

Stirred bioreactor experiment

Batch cultivation was carried out in a 3.5 L stirred bioreactor (Chemap). Temperature, aeration and agitation were set at the following cultivation conditions: 28 °C, 2.5 L air/L waste water/min and 250 r.p.m., respectively. The bioreactor was filled with 2.25 L of filtered waste water, sterilized at 121 °C for 20 min and 10 % of yeast inoculum was added. Samples were taken every 6 hours for COD, TOC, biomass and fructose determination, while *pH* and pO_2 were monitored on-line.

Analytical methods

Biomass dry weight: fungal or yeast biomass were separated from waste water by filtration (Sartorious 0.2 μm), dried at 110 °C for 8 hours and determined gravimetrically.

Total solids, ash and Kjeldahl nitrogen were determined according to AOAC methods *(8)*.

Chemical oxygen demand (COD) of the samples was determined by following the APHA dichromate method *(9)*.

Biochemical oxygen demand (BOD) was estimated by incubating the samples for 5 days at 20 °C according to the APHA method *(9)*.

Ammoniacal nitrogen was determined according to the AOAC titrimetric method *(8)*.

Sugars, erythritol and mannitol analyses were performed by HPLC with an Eurocat Ca column at 65 °C: mobile phase H_2O; flow rate 0.6 mL/min; detector differential-refractometer.

Phosphates were quantitatively estimated using a Merck Microquant 14846 test kit.

Ergot alkaloids were analyzed by HPLC using a Lichrosorb RP column at room temperature; mobile phase 48 % acetonitrile in water with ammonium carbonate (2 g/L); flow rate 1–1.5 mL/min; fluorescence detector (λ_{ex} =254 nm; λ_{em}=425 nm).

Carbohydrates were determined with anthrone reagent *(10)*.

Ethyl acetate and ethanol were determined by gas chromatography on a Carbopack C column with a temperature gradient of 4–8 °C/min; oven temperature program 60–80 °C, 2–4 min, 170 °C, 20 min, injector temperature 110–190 °C and detector temperature 190–250 °C.

Total organic carbon (TOC) was measured using a TOC Shimadzu Analyzer (Model 5000 A).

Results and Discussion

The chemical composition of waste water and its filtrate is presented in Table 1. Variations in chemical characteristics of waste water originated mainly from various media prepared for the production of specific types of ergot alkaloids and diverse metabolism of various production strains of *Claviceps purpurea*. In our study we used waste water with a COD value ranging from 35 000 to 39 000 mg/L after filtration. The filtration of waste water reduces the COD value by elimination of fungal biomass and other solids.

Table 1. Composition of waste water and its filtrate from 6 ergot alkaloid production batches

Parameters	Waste water	Filtrate
γ(fungal biomass d.w.)/g L^{-1}	6–16	0
γ(ergot alkaloids)/mg L^{-1}	53[1]	3–20
γ(carbohydrates)/g L^{-1}	27–39	20–34
γ(ethyl acetate)/%	0.27–0.61	0.10–0.50
γ(ethanol)/%	0.35–1.2	0.10–0.40
γ(phosphate)/g L^{-1}	1.2–1.8	1.0–1.4
γ(NH_3–nitrogen)/mg L^{-1}	7–50	5–55
γ(total nitrogen; Kjeldahl)/g L^{-1}	0.5–0.9	0.2–0.4
γ(total solids)/g/L^{-1}	25–50	15–27
γ(total ash)/g L^{-1}	8–17	7–14
pH	5.5–7	5.5–7
γ(COD)/mg L^{-1}	50 000–68 000	13 000–42 000
γ(BOD_5)/mg L^{-1}	21 000–26 000	12 000–20 000

[1] Data from one sampling only

Table 2. Selected yeast strains from the Culture Collection of Industrial Microorganisms (ZIM) according to a COD reduction in the shake flask experiment after 72 hours of incubation in filtered waste water at 28 °C

Yeast strain	ZIM	COD reduct. %	*w*(biomass d.w.) g/L
Kluyveromyces marxianus	1	27	2.1
Torulaspora pretoriensis	3	43	5.9
Schizoblastosporion starkey	4	30	2.7
Sympodiomyces parvus	8	48	3.4
Candida crusei	11	46	4.1
Sporobolomyces roseus	10	53	4.4
Candida catenulata	13	51	3.9
Zygosaccharomyces rouxi	15	46	3.3
Zygohansenula californica	16	56	5.0
Endomycopsis javanensis	22	36	2.9
Endomyces venalis	26	38	3.1
Pichia anomala	**31**	**64**	**7.1**
Lodderomyces elongiosporus	55	56	8.3
Sterigmatomyces indicus	56	56	7.5
Pichia kluyveri	**60**	**61**	**7.6**
Geotrichum penicillatum	61	51	3.3
Candida vini	62	48	3.7
Candida trigonopsoides	63	42	2.6
Candida methilica	64	45	3.3
Debaryomyces kloeckeri	65	24	2.3
Pichia jadinii	**70**	**60**	**4.0**
Candida utilis	71	52	3.2
Bullera tsugae	72	51	5.6
Candida pseudotropicalis	75	57	2.9
Metschnikowia reukaufi	78	35	4.3
Candida armenti	79	21	1.0
Saccharomyces cerevisiae	1235	33	1.5
control sample		10	0
initial COD of control sample/ mg L^{-1}		38 900	

Table 3. Selected yeast strains isolated from the ergot alkaloid waste water collecting tank of Lek Pharmaceutical & Chemical Company, Ljubljana, according to a COD reduction in the shake flask experiment after 72 hours of incubation in filtered waste water at 28 °C

Isolated yeast strain	COD reduction %	*w*(biomass d.w.) g/L
ZIM 83	**67**	**11.1**
ZIM 84	26	2.1
ZIM 85	**72**	**12.1**
ZIM 86	32	3.9
ZIM 87	28	2.4
ZIM 88	**73**	**12.5**
ZIM 89	28	2.6
ZIM 90	15	1.8
ZIM 91	43	2.7
ZIM 92	**68**	**12.0**
ZIM 93	**74**	**12.2**
ZIM 94	**72**	**13.2**
ZIM 95	**71**	**11.7**
ZIM 96	**68**	**11.9**
ZIM 99	**70**	**12.3**
ZIM 419	**67**	**11.6**
control sample	6	0
initial COD of control/mg L^{-1}	37 000	

Screening of yeasts in shake flask cultivation

Preliminary screening of 60 yeast strains from the Culture Collection of Industrial Microorganisms (ZIM) was performed on waste water and its filtrate solidified with agar (the results are not shown). It was found that 27 yeast strains had the capability of growing on waste water and they were further studied in shake flask experiments. Additionally, 16 yeast strains from the ergot alkaloid waste water collecting tank were isolated and they were also examined in the same experiment.

The main criterion for yeast screening was efficient COD reduction of filtered waste water. Regarding this criterion, only 3 out of 27 yeast strains from the ZIM Culture Collection belonging to the *Pichia* genus showed a COD reduction capacity greater than 60 % within 72 hours (Table 2). The results given in Table 3 showed that 10 out of 16 isolated yeast strains reduced COD efficiently (68–74 % COD reduction was reached within 72 hours). Identification of these yeast strains showed that they belonged to the species *Pichia anomala*. Further characterization by kariotyping with pulsed field gel electrophoresis showed that 6 out of 10 yeast strains had different electrophoretic patterns *(11)*. The next six isolated strains (Table 3) revealed poor growth reflected in low dry biomass accumulation (1.8–3.9 g/L) as well as COD reduction (15–41 %).

Comparing biomass production among yeast isolates and yeast strains from the ZIM Culture Collection showing efficient COD reduction revealed that a higher amount of biomass was obtained with the strains obtained by spontaneous selection pressure in the waste wa-

ter collecting tank. This suggested that isolated yeast strains were capable of utilizing organic compounds, which remained in the media after *Claviceps purpurea* growth.

Cultivation of yeast isolates in airlift bioreactor

Further screening of yeast strains was carried out in a 2 L airlift bioreactor where COD level (Fig. 1) and biomass production (Fig. 2) were monitored during the bioprocess. All 10 previously selected yeast isolates gave similar results for COD reduction and 3 of them, ZIM 88, ZIM 95 and ZIM 419, are presented in Fig. 1.

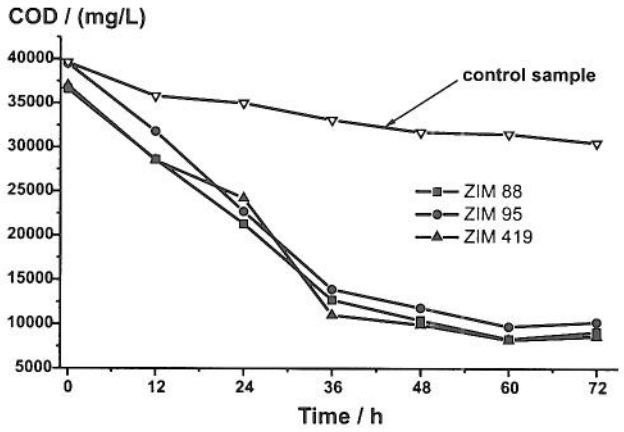

Fig. 1. Reduction of COD in filtered waste water with 3 selected yeast isolates cultivated in the 2 L airlift bioreactor

The cultivation of all yeast isolates in the airlift bioreactor confirmed the previous results from the shake culture experiment and showed improved COD removal efficiencies, which were 68–80 % after 60 hours when the majority of isolates reached the maximum COD reduction. This could be attributed to the better aeration conditions in the bioreactor than in the shake flasks. The COD reduction found in our study corresponds to the range reported in the literature for aerobic treatment of polysaccharide-containing waste waters *(12–14)*. A similar study of aerobic waste water treatment from the antibiotics industry, using mixed microbial culture, showed 96 % COD reduction *(15)*. However, this process had a 13–14 times lower initial COD load (2 500 mg/L) than waste water in our study. Yiao *(16)* reported a 69 % reduction of initial COD (105 100 mg/L) during treatment of waste water from monosodium glutamate manufacturing using the yeast *Candida tropicalis*.

When comparing growth dynamics of selected yeast strains it was evident that ZIM 419 showed a longer exponential growth phase (up to 48 hours) with higher biomass accumulation (up to 5 g/L) than the other isolated strains which entered the stationary phase after 36 hours, with final biomass production ranging from 3–4 g/L of dry weight (Fig. 2).

Cultivation of isolated yeast ZIM 419 in stirred bioreactor

The possibility of scale-up was investigated and according to this strategy, cultivation in a 3.5 L laboratory stirred bioreactor was performed. During 37 hours of

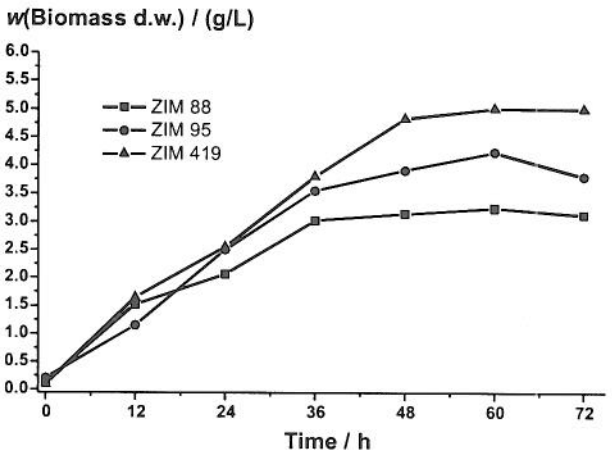

Fig. 2. The biomass production by 3 selected yeast isolates cultivated in the 2 L airlift bioreactor

the cultivation of yeast isolate ZIM 419 changes in *pH*, pO_2, COD, fructose concentration, biomass accumulation and TOC were followed, (Fig. 3). When analyzing carbon sources of filtered waste water with 36 900 mg/L of initial COD, the main carbohydrate found was fructose, which represents 50 % of the COD load. The rest of the COD load was erythritol and mannitol in the concentration range 5–6 g/L, glucose and sucrose in the concentration under 2 g/L and other unknown organic compounds (data not shown). Fig. 3 shows that fructose was completely consumed within 26 hours, when total COD reduction reached 74 %. A further 7 % of COD reduction was due to yeast metabolic activity and consumption of other organic compounds in waste water. At the beginning of the bioprocess 12.8 g/L TOC was present in waste water, which decreased to 2.6 g/L within 37 hours. The *pH* value of waste water was stable to the end of the logarithmic growth phase, when it began to increase from 5.4 to 6.2. When analyzing the metabolic activity of yeast through pO_2 concentration in the media, it was found that pO_2 followed yeast growth dynamics.

Conclusions

The present study serves as the model study for yeasts which could be capable of efficient COD reduction of waste water from ergot alkaloid production. Waste water contained a high concentration of organic matter which was easily degradable by yeasts. The ratio of yeasts successful in COD reduction is in favor of the yeast isolates from waste water compared to the yeasts from other environmental niches deposited in the ZIM Culture Collection. Yeast isolates, identified as *Pichia anomala* strains, were capable of efficient COD reduction (68–80 %) after 60 hours of the shake culture experiment. Cultivation of the isolate ZIM 419 in a 3.5 L stirred bioreactor indicates that 81 % of initial COD was reduced within 37 hours. The latter case indicates improved bioprocess productivity and the possibility of direct employment of this system on an industrial scale for treatment of drain waste water. Biomass harvested

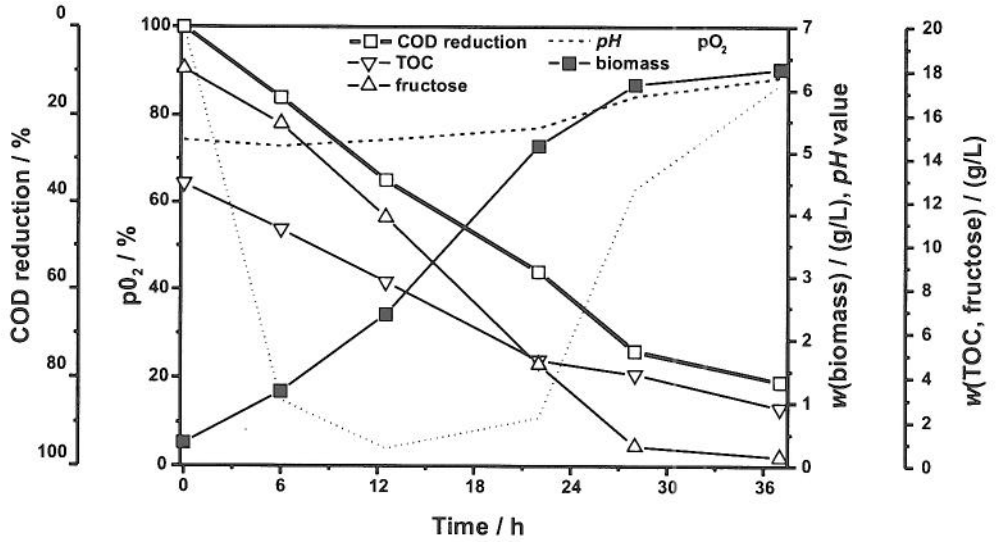

Fig. 3. Dynamics of *pH*, pO_2, COD, TOC, fructose changes and biomass production during cultivation of isolated yeast ZIM 419, identified as the *Pichia anomala* strain, in the 3.5 L stirred bioreactor

after wastetreatment could serve as a valuable by-product used for animal feed.

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Mogućnosti rasta kvasaca i smanjenje kemijske potrošnje kisika u otpadnoj vodi iz proizvodnje ergot alkaloida

Sažetak

Proveden je izbor sojeva kvasaca sposobnih da rastu u otpadnim vodama iz proizvodnje ergot alkaloida, te ispitana njihova sposobnost za smanjenje kemijske potrošnje kisika. Djelotvorno smanjenje kemijske potrošnje kisika u filtriranoj otpadnoj vodi bio je glavni kriterij pri izboru 27 prethodno odabranih sojeva kvasaca iz Zbirke kultura industrijskih mikroorganizama (ZIM) u Ljubljani. Ispitivanje je obuhvatilo i 16 izoliranih sojeva kvasaca koji su potjecali iz otpadne vode. Dobiveni rezultati pokazuju da tri soja iz zbirke ZIM smanjuju kemijsku potrošnju kisika preko 60 %, a 10 od 16 izoliranih sojeva kvasaca snizuju 68–74 % početne kemijske potrošnje kisika unutar 72 sata. Konačni odabir sojeva proveden je u bioreaktoru s aeracijom, kapaciteta 2 L. Nađeno je da se 10 izolata kvasaca, utvrđenih kao Pichia anomala, mogu koristiti za smanjenje kemijske potrošnje kisika u otpadnoj vodi iz proizvodnje ergot alkaloida. Jedan od izoliranih kvasaca, ZIM419, bio je uzgojen u bioreaktoru s miješalicom veličine 3,5 L. S njim je postignuto 81 %-tno smanjenje početne kemijske potrošnje kisika unutar 37 sati.