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THE USE OF YEAST MANUFACTURE LIQUID WASTES IN THE PRODUCTION OF BIOFERTILIZERS AND PHYTOHORMONES

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ABSTRACT

The cost and composition of the nutrient medium are considered limiting factors for the production of bacterial inocula. At the same time, baker's yeast factories get rid of their liquid wastes in River Nile or water drains. The analysis of such wastes indicated their richness in various minerals, amino acids, vitamins and yeast cells. The recommended media used for *Azotobacter vinelandii*, *Azospirillum brasilense*, *Bacillus polymyxa*, *Klebsiella pneumoniae* and auxin over-producing mutant (*Azospirillum brasilense* FT) were compared with optimized yeast liquid waste (OYW) in respect to the production of both bacterial inocula and phytohormones. Data showed that *Klebsiella pneumoniae* and *Azospirillum brasilense* FT grew well in the optimized yeast liquid waste medium compared to other diazotrophs. It was also found that *Azospirillum brasilense* FT mutant produced high quantities of extracellular indoleacetic acid (IAA) and tryptophol (TOL) in both recommended medium and the optimized yeast liquid waste medium. The production of both compounds was increased at the stationary phase of the culture. These data suggested that, optimized yeast liquid waste could be used as an alternative medium for biomass and phytohormones production of associative diazotrophs.

INTRODUCTION

There is a great deal of interest in creating efficient associations between agronomically important plants, particularly cereals, and associative N_2-fixing bacteria (diazotrophs). The long-term objectives of such studies are to introduce the ability to fix N_2 into plants, which could enhance plant growth and reduce the dependence of the plants on N fertilizer applications, or to enhance the efficiency of applied fertilizer (fertilizer efficiency), resulting in saving fertilizer. These objectives could have both economic and environmental significance. Intensive application of chemical fertilizers imposes undesirable consequences of pollution besides to the ever-increasing costs of such chemicals. Many reports have been published on beneficial effects of diazotrophs inoculation on plant growth (Sumner 1990; Gunarto *et al.* 1998; El-Khawas *et al.* 2000). Diazotrophs are found to produce and release a broad spectrum of plant growth regulators such as auxins, several gibberellins, and cytokinnins (Tien *et al.* 1979; El-Khawas and Adachi 1999). Responses of cereal plants to inoculation with associative N_2-fixing bacteria were explained as an effect of plant growth regulators released by such microorganisms (Salamone *et al.* 1997; El-Khawas 1990;). Costs of bacterial biomass production depend on prices of production medium particularly substrates used as carbon sources. Therefore, the possibility of replacing the recommended media for biomass and phytohormones

production of various diazotrophs by yeast manufacture liquid wastes as a cheap medium was investigated.

MATERIALS AND METHODS

Bacterial strains

Strains of *Azotobacter vinelandii* ATCC 12837, *Azospirillum brasilense* ATCC 29710, *Azospirillum brasilense* FT (an IAA-overproducing mutant of *A. brasilense* ATCC 29710, El-Khawas 1990), *Bacillus polymyxa* ATCC 842 and *Klebsiella pneumoniae* ATCC 13883 were used.

Media

Modified N- deficient medium of Yensen, yeast extract manitol (YEM) medium, nitrogen free glucose (NFG) medium and nitrogen free malate (NFM) medium were used as recommended media (Deutsche Sammlung von Mikroorganismen, DSM, 1977).

Yeast manufacture liquid wastes

The diagram (Fig. 1) shows the various stages of wastes produced during baker's yeast production at Yeast Factory, Grand Cairo Bakers, El-Salam City. The raw waste (waste 1) and waste 2 (after first wash) were used for growing the different diazotrophs in comparison with the recommended media.

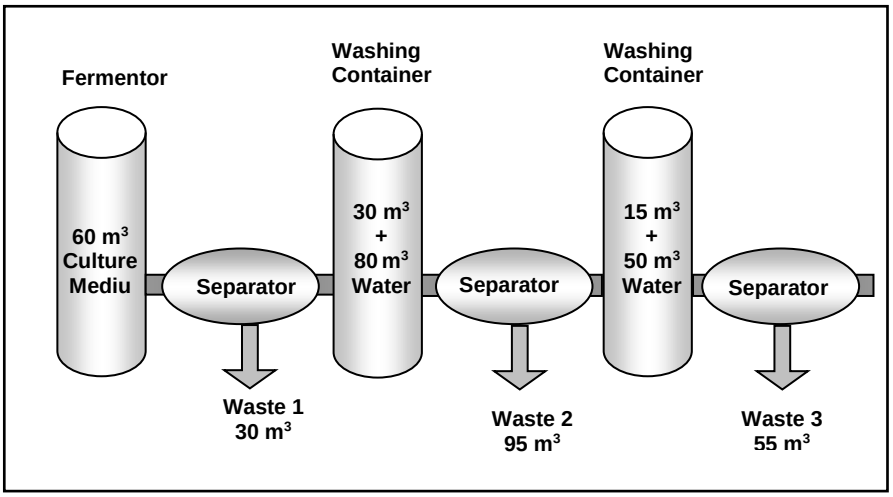

Figure 1: Diagram for various stages of wastes during baker's yeast production at Yeast Factory, Grand Cairo Bakers, El-Salam City.

Optimization of the yeast liquid wastes

Waste 1 and waste 2 were mixed with the rate of 1:1 without removing yeast cells and the pH was adjusted at 7.0. The autoclaved mixture was used as optimized yeast waste (OYW).

Determination of auxins

Extracts from the cultured supernatant of *Azospirillum brasilense* FT for different incubation periods were partitioned into two fractions and analyzed by using high performance liquid chromatography (HPLC) according to El-Khawas and Adachi (1999). The supernatants (30 ml) of stationary phase cultures were adjusted to pH 8.6 with 1% NaOH and partitioned three times with equal volumes of ethyl acetate. The combined ethyl acetate fraction were evaporated to dryness and held for further purification. The aqueous phase was adjusted to pH 2.8 with 1% HCl and partitioned three times with equal volumes of ethyl acetate, while the remaining aqueous phase was discarded. The combined acidic ethyl acetate phase was reduced to 5 ml volume (Fraction1) and used for HPLC determination of acidic auxins. The dried basic ethyl acetate fraction was dissolved in 80% methanol, then methanol was evaporated under vacuum, leaving an aqueous phase, which was adjusted to pH 2.8 and partitioned three times with 25 ml of ethyl acetate. The ethyl acetate phase was combined (Fraction 2), reduced to 5ml volume and used for HPLC determination of neutral auxins.

Mobile phases and standards used in the study

Tow different optimized mobile phases e.g., isopropanol/H_2O: 12.7/87.3 containing 1.9mM citrate pH 4.35, and gradient 10-70% methanol in 10 mM aqueous acetic acid were used. The standards e.g., indole-3-acetic acid (IAA), indole-3-acetaldehyde (IAAld), indole-3-pyruvic acid (IPyA), and tryptophol (Indole-3- ethanol, TOL) were used as standards for identification of HPLC peaks.

Chemical analysis

Crud protein was determined by automated method using Kjel-Tec automatic as described in the A.O.A.C. (1998). Sodium, potassium, calcium, phosphorus and other minerals were determined according to the method described in the A.P.H.A (1992) using atomic absorption spectrophotometer, 3300 Perker-Elmer. Amino acids were determined by Beckman amino acids analyzer model 7300 and data system model 7000 according to the method of Winder and Eggum (1966). Vitamins were determined by high-performance liquid chromatography (HPLC) and post-column derivatization according to the method of Bognar (1992).

RESULTS AND DISCUSSION

Chemical analysis of the two yeast liquid wastes (W1 and W2)

Table (1) shows the chemical analysis of the two yeast liquid wastes and other recommended media used in the study. Data revealed that waste 1 contains more minerals than waste 2. When the concentrations of the elements in the two wastes were compared with recommended media, waste 1 contains high concentration of elements specially potassium, calcium, magnesium, iron and manganese. However, waste 2 contains lower concentration of elements compared with some of the recommended media. Furthermore, the concentration of carbon and nitrogen were higher in the waste 1 than waste 2 and recommended media.

Table 1: Minerals content (mg l^{-1}), carbon and nitrogen (%) in yeast wastes (W1 & W2) and other recommended media.

Elements	Yeast wastes		Recommended media			
	W 1	W 2	Yensen	YEM	NFG	NFM
P	115	45	188	89	178	109
K	1450	152	236	112	224	137
Ca	110	9	9	-	3	3
Mg	1310	123	50	20	20	20
Na	75	6	-	39	-	39
Fe	65	8	10	-	10	2
S	-	-	85	26	26	26
Mo	-	-	22	-	1	1
Mn	35	-	-	-	1	-
Carbon (%)	2.6 as total carbohydrate 0.3 as reducing sugar	0.7	1.0 as glucose	1.0 as sucrose	1.0 as glucose	0.5 as malic acid
Nitrogen (%)	0.8 as total nitrogen	0.3	0.1 NH_4NO_3	0.1 NH_4NO_3	0.1 NH_4NO_3	0.1 NH_4NO_3
	0.2 as non protein nitrogen	0.07				

Data in Tables 2 and 3 revealed that, the two wastes contained comparable amount of amino acids and vitamins. This is could be due to the presence of nearly the same number of viable yeast cells in both wastes. It is quite obvious that yeast manufacture liquid wastes are reaches in various minerals, amino acids, vitamins and yeast cells. The presence of yeast cells in the wastes offered an excellent source four vitamins and growth factors

which are required for the growth of most microorganisms (Deutsche Sammlung von Mikroorganismen, DSM, 1977).

Table 2. Amino acids (mg l^{-1}) in the two yeast liquid wastes.

Amino acid	Wast1	Waste 2	AMINO ACID	Waste 1	Waste 2
Asparatic	1386	1120	Isoleucine	70	81
Threonine	91	85	Leucine	112	96
Serine	119	112	Tyrosine	21	24
Glutamic	1155	1010	Phenylalanine	42	38
Proline	77	84	Histidine	21	16
Glysine	147	122	Lysine	70	62
Alanine	210	198	Valine	91	84

Table 3. Vitamins and viable yeast cells of both yeast wastes

Wastes	Vitamins (μg l^{-1})		Number of viable yeast cells (X 10^4 cfu l^{-1})
	B2	B6	
Waste 1	300	125	175
Waste 2	320	110	142

Growth curves of *K. pneumoniae* grown in the two wastes

In a primary experiment, the growth curves of *K. pneumoniae* in the two wastes and on mixture of both of them were investigated. The growth of *K. pneumoniae* was similar in both wastes and reached to 10^8 cells ml^{-1} culture after 48 h. of incubation (Fig. 2). However, when *K. pneumoniae* was grown in mixture of waste 1 and waste 2 at the rate of 1:1, the growth was increased and reached to 10^{10} cells ml^{-1} culture after 36 h.

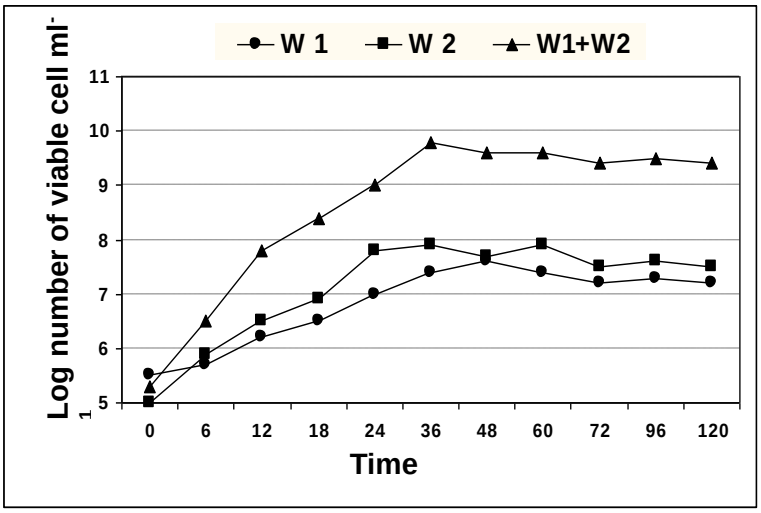

Figure 2: Growth curves of *K. pneumoniae* grown in yeast liquid wastes

Therefore, to optimize the liquid wastes to obtain best medium for growing the different diazotrophs, it must be mixed together at the rate of 1:1 without removing yeast cells and adjusted the pH at 7.0. The autoclaved mixture was used as optimized yeast waste medium (OYW).

Growth curves of diazotrophs grown in optimized yeast liquid waste medium (OYW)

The growth performance of some diazotrophs in optimized yeast waste medium (OYW) was compared with the same medium supplemented with 0.5% of carbon sources, and the recommended media of such organisms (figures 3 and 4). Generally, data showed that *Klebsiella*

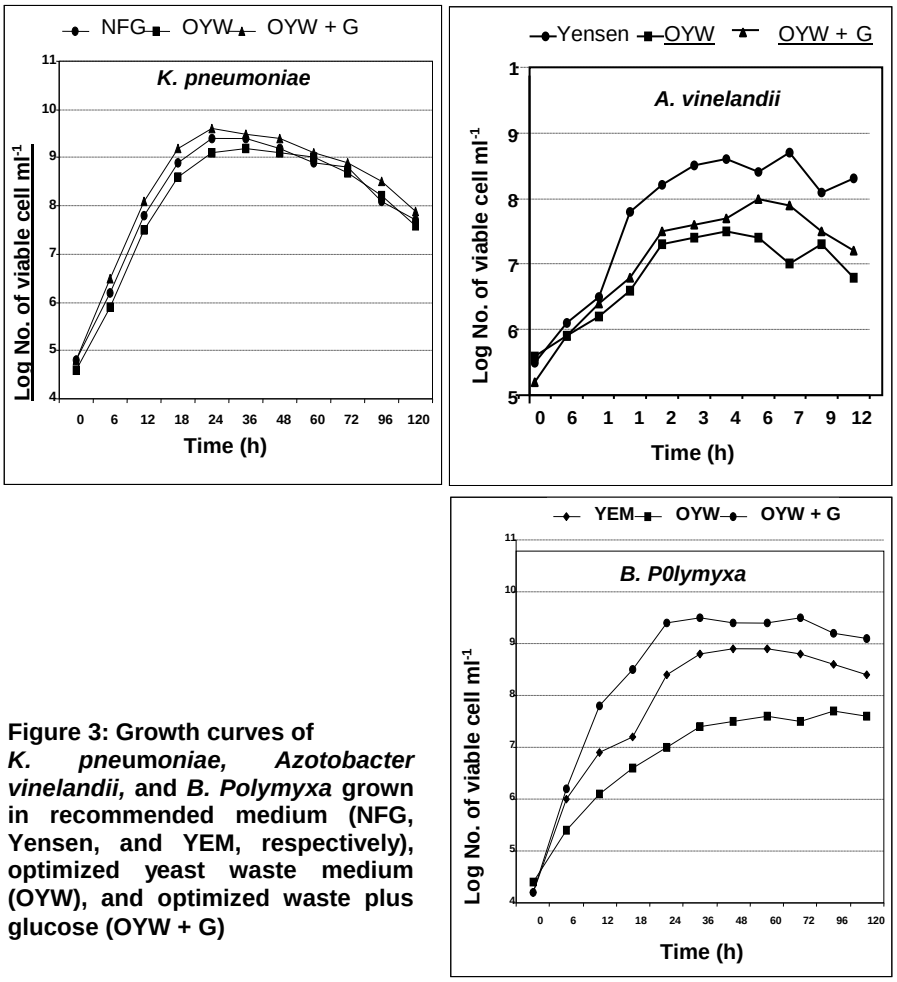

Figure 3: Growth curves of *K. pneumoniae, Azotobacter vinelandii,* and *B. Polymyxa* grown in recommended medium (NFG, Yensen, and YEM, respectively), optimized yeast waste medium (OYW), and optimized waste plus glucose (OYW + G)

pneumoniae grew well in the three different media compared to other diazotrophs. When glucose was added to optimized yeast waste medium, a

slight increase in viable cell counts of K. *pneumoniae* was achieved (Fig. 3). The growth of *Azotobacter vinelandii* in OYW medium was week compared with other diazotrophs. However, when glucose was added to the medium, the growth rate was increased but still lower than the recommended medium (Fig. 3). With regard to *B. polymyxa*, the growth was week on OYW medium. Addition of glucose to the medium enhanced the growth until exceeded their numbers in the recommended medium (Fig. 3).

Regarding to *Azospirillum*, two strains (wild type of *Azospirillum brasilense* ATCC 29710 and its mutant *Azospirillum brasilense* FT) were used. The growth of *A. brasilense* FT mutant was better than *A. brasilense* wild type strain when grown on OYW medium. However, when malic acid was added to the OYW medium, the growth rate of *A. brasilense* wild type strain increased and reached to similar growth rate as the growth in the recommended medium (Fig. 4).

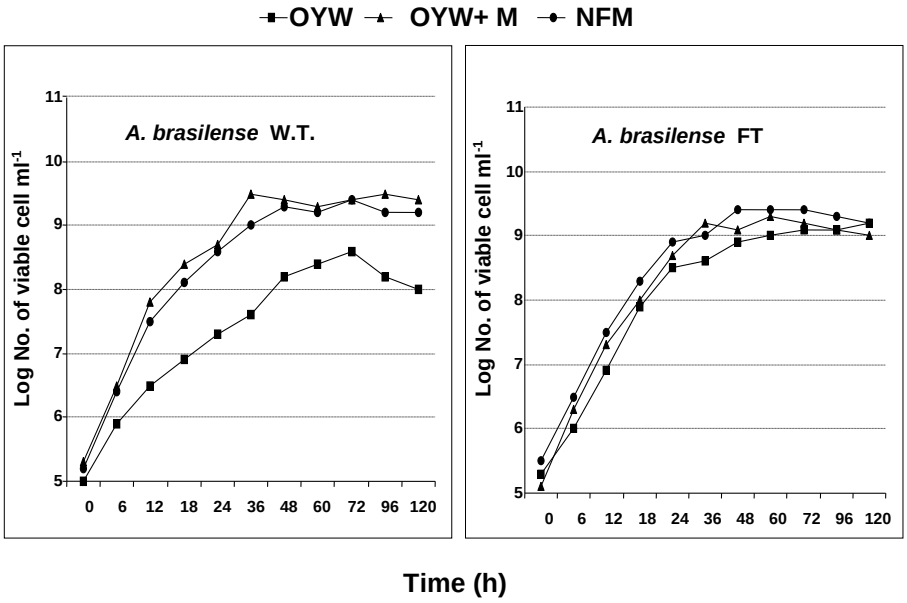

Figure 4: Growth curves of *A. brasilense* wild type strain (W.T.) and FT mutant grown in optimized yeast waste medium (OYW), optimized waste plus malate (OYW+M), and recommended medium (NFM)

Excretion of auxins in optimized yeast waste and recommended media

Figure 5 represents the production of different auxins (indoleacetic acid and related compounds) by *Azospirillum brasilense* FT in optimized yeast waste medium as well as N-deficient malate culture medium. After the strain was grown in both culture media, extracts from the cultured supernatant were partitioned and analyzed by using high-performance liquid

chromatography (HPLC). Data revealed that both culture media contained high quantities of extracellular indoleacetic acid (IAA) and tryptophol (TOL). The production of both compounds was increased at the stationary phase of the growth (Fig. 6). On the other hand, very low levels of indoleacetaldehyde (IAAld) and indolepyruvate (IpyA) were identified in optimized yeast waste culture medium only (Fig. 5). Similar results were reported in *Azospirillun lipoferum* and *Azospirillum brasilense* (Abd El-Salam and Klingmuller 1987, Dosselaere *et al.* 1997, El-Khawas and Adachi 1999). They indicated that both *Azospirillun lipoferum* and *Azospirillum brasilense* could produce IAA, TOL and IpyA when these microorganisms were cultured in their respective media.

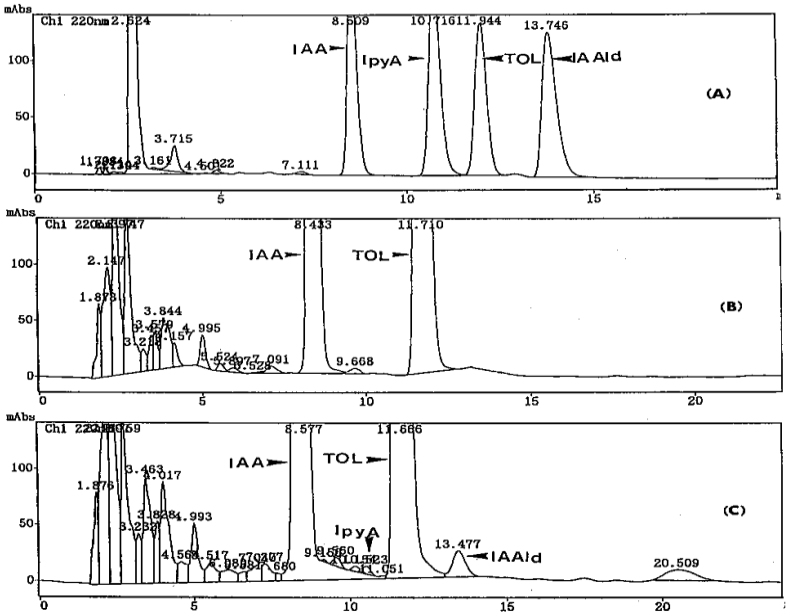

Figure 5: HPLC analysis of different auxins standard (A) and supernatants of 48 h. old cultures of *Azospirillum brasilense* Ft grown in N-deficient malate culture medium (B) and optimized yeast waste medium (C). IAA, indoleacetic acid; TOL, tryptophol; IAAld, indoleacetaldehyde and IpyA, indolepyruvate.

Many industrial by-products and wastes have been used as growth substrates for microbial biomass and production of economically valuable microbial products. Several investigators employed many wastes as media for diazotrophs. Bioardi and Ertola (1985) showed that a malt sprout is a cheap product, which could be an adequate component of media for rhizobia. Raw whey as well as deproteinized whey were successfully used as a cheap medium for rhizobia and azospirilla (Bissonnette, *et al.* 1986; Omar, *et al.* 2000). On the other hand, Martinez-Toledo et al. (1995) showed that alpechin (wastewater from olive oil mills) could be utilized by

Azotobacter as a cheap substrate for growth and producing poly-B-hydroxbutyrate (PHB).

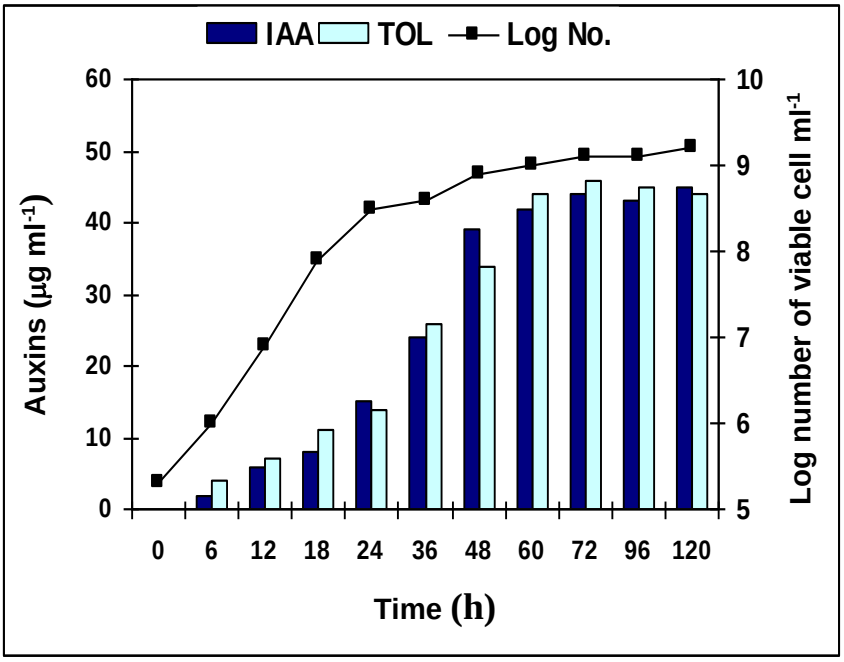

Figure 6: Excretion of auxins (IAA and TOL) and growth curve of *Azospirillum brasilense* FT mutant grown in optimized yeast waste medium.

Generally, it could be suggested that, it is feasible to apply the optimized yeast liquid waste (OYW) as an alternative medium for biomass and phytohormones production of associative diazotrophs.

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استخدام المخلف السائل لمصانع الخميرة في إنتاج الأسمدة الحيوية والهرمونات النباتية

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تعتبر تكاليف وتركيب البيئة الغذائية من أهم العوامل المحددة لإنتاج اللقاحات البكتيرية. في الوقت الذي تتخلص مصانع إنتاج خميرة الخباز من المخلفات السائلة بإلقائها في مياه النيل ، ومياه البحار أو المجارى مما يتسبب عنه أضرارا للبيئة . وبتحليل هذا المخلف السائل وجد انه يحتوى على كثير من العناصر المعدنية، والأحماض الأمينية و الفيتامينات . لذلك كانت فكرة هذا البحث هي كيفية تعديل هذا المخلف لإعادة استخدامه كبيئة غذائية لإنتاج الأسمدة الحيوية والهرمونات النباتية. استخدم في هذا البحث مجموعة من مثبتات النتروجين الجوى (ازوتوباكتر فينلديای، ازوسبيريللم برازيلنس، باسيليس بوليميكسا وكليبسيلا نيمونيا) بالإضافة إلى إحدى الطفرات العالية في إنتاج الآوكسينات (ازوسبيريللم برازيلنس FT).

أوضحت النتائج أن كل من كليبسيلا نيمونيا وازوسبيريللم برازيلنس FT ينمو جيدا على المخلف المعدل مقارنة بباقي أنواع البكتريا المستخدمة ، وان درجة النمو على المخلف تقترب بنسبة كبيرة من درجة النمو على البيئات المتخصصة. وقد وجد أيضا أن الطفرة FT تنتج كميات كبيرة من أو كسينات ألا ندول اسيتيك اسيد والتربتوفول عند نموها على كل من المخلف المعدل والبيئة المتخصصة .

عموما، توكد نتائج هذا البحث إمكانية الاستفادة من المخلف السائل لمصانع إنتاج الخميرة وذلك بإعادة استخدامه في إنتاج الآسمده الحيوية والهرمونات النباتية .