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High pyrazine production by *Bacillus subtilis* in solid substrate fermentation on ground soybeans

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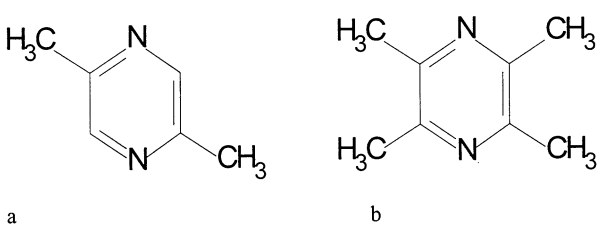
Abstract

2,5-Dimethylpyrazine (2,5-DMP) and tetramethylpyrazine (TTMP) were produced using *Bacillus subtilis* IFO 3013 grown in solid substrate conditions using ground soybeans suspended in water. Optimization studies showed that the best way to produce the two above aroma compounds involved massive enrichment of the medium with L-threonine and acetoin. The amino acid allowed 2,5-DMP formation and was added at 40 g/l at the beginning of a process, while acetoin was the precursor of TTMP and had to be added at 60 g/l only after 2,5-DMP production was terminated. The optimal cultivation temperature was 40°C, pH had to be monitored at 7.5, and aeration rate had to be higher than 0.1 VVM with a volumetric oxygen tranfer coefficient k_La close to 180 h^{-1}. These conditions allowed recovery of 2 g/l total pyrazines, that demonstrated the efficiency of this approach.

Keywords: Aroma; Pyrazines; *Bacillus subtilis*; Soybean; Solid substrate fermentation

1. Introduction

An increase in the production of processed foods by industrial methods has resulted in an expanding market for additives such as flavourings. In this area, consumers generally prefer additives exhibiting a natural label and food manufacturers often use these items although they are generally more expensive than the corresponding chemical compounds. Fermentation or biotransformation processes are claimed to be an useful tool for the production of this kind of natural components [1].



a b

Fig. 1. Structural formulae of 2,5-dimethylpyrazine (a) and of tetramethylpyrazine (b).

Alkylpyrazines are heterocyclic, nitrogen containing molecules found in a wide variety of foods. They are responsible for different flavours, according to the nature of the alkyl substituents, but are generally considered as giving nutty, roasty and toasty tonalities [2–4]. These characteristics make them useful for use as additives for flavouring in the food industry [4]. 2,5-Dimethylpyrazine and tetramethylpyrazine (Fig. 1) are the main pyrazines detected in many cocoa bean- or soybean-based fermented foods, where they significantly contribute to their aroma [5,6].

The first evidence that microorganisms were able to synthesize pyrazines was provided by 1962 by Kosuge and coworkers [7] who showed that tetramethylpyrazine could be produced by *Bacillus subtilis*. Since this time, several other microorganisms able to synthesize different alkylpyrazines have been discovered [2,4]. However, attempts to use them has until now resulted in metabolite concentrations too low to allow industrial applications of these processes [8].

This situation has been modified by a recent work [9], in which *B. subtilis* IFO 3013 was grown on soybeans

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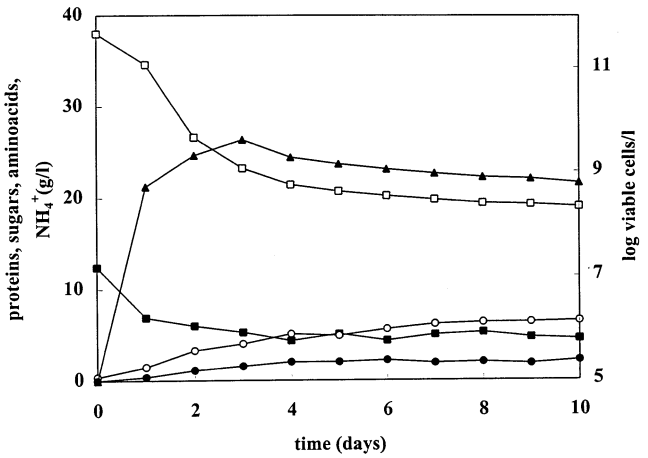

Fig. 2. Time-course of total proteins (□), soluble aminoacids (○), total sugars (■), NH_4^+ (●), and viable cells (▲) concentrations during cultivation of *B. subtilis* IFO 3013 on standard ground soybeans. Experiment carried out in an Erlenmeyer flask, inoculation level 0.5×10^5 cells/ml, temperature 27°C, stirring speed 250 rpm.

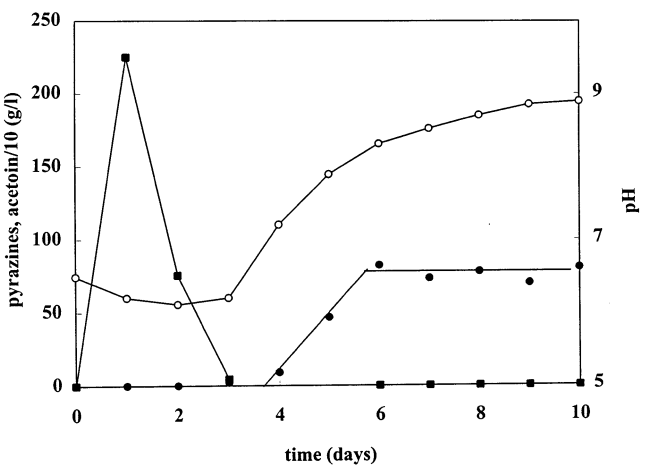

Fig. 3. Acetoin (■), total pyrazines (●), and pH (○) plotted against time during cultivation on standard ground soybeans. Experimental conditions are as in Fig. 2 legend.

using solid-state fermentation techniques. Results clearly showed that, with this strain, threonine was the precursor of 2,5-dimethylpyrazine while tetramethypyrazine was obtained from acetoin and ammonia. Enrichment of the substrate with threonine resulted in the synthesis of 0.85 g metabolite/kg IDW (initial dry weight) while supplementation with acetoin gave 2.5 g metabolite/kg IDW. This process could in fact be considered as a modified Itohiki-Natto production system, allowing a very strong increase in aroma synthesis.

It is now often recognized that metabolite production by solid-state cultivation rarely competes with submerged techniques, generally due to water limitations [10]. The aim of the present work was to investigate the results obtained when the above system was adapted to submerged techniques using ground soybeans; i.e. when the cultivation technique moves from solid state to solid substrate fermentation [11].

2. Materials and methods

2.1. Microorganism

Bacillus subtilis IFO 3013, which had been previously selected for pyrazine production in solid state cultivation on soybeans [9], was used throughout this work. It was conserved by periodic replications on tryptone/soy/agar (Difco Laboratories, Detroit, Michigan) in Petri dishes.

2.2. Inoculum preparation

Bacillus subtilis was cultured at 27°C for 20 h on the above medium in Petri dishes. Cells were then recovered by soaking each Petri dish content with 5 ml sterile distilled water [9].

2.3. Cultivation medium

Dehulled yellow whole soybeans (100 g, dry weight), obtained from a dietary shop, were soaked in 500 ml distilled water at 4°C for 15 h. They were ground in 1 l (final volume) distilled water using an Ultra Turrax blender. Alternatively, this medium was enriched with compounds such as acetoin (0–80 g/l), L-threonine (0–100 g/l), or both. The resulting mixture was autoclaved at 120°C for 20 min. Inoculation was performed at 2% (V/V) level, giving an initial bacterial loading ranging from 5×10^4 to 5×10^5 cells/ml.

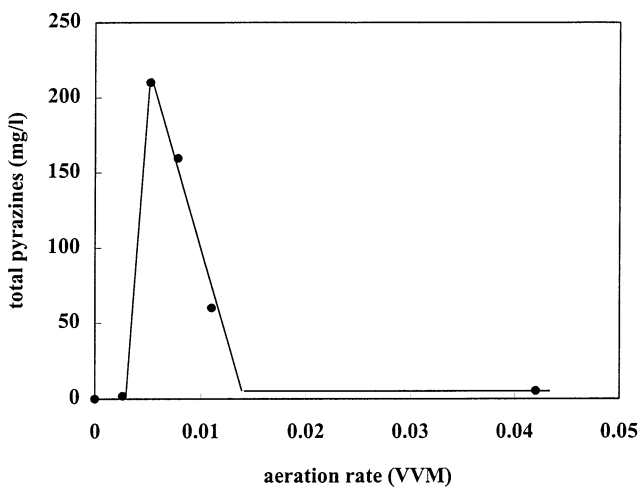

Fig. 4. Influence of the aeration rate on pyrazine synthesis with standard ground soybeans in an aerated, stirred (700 rpm) bioreactor at 27°C.

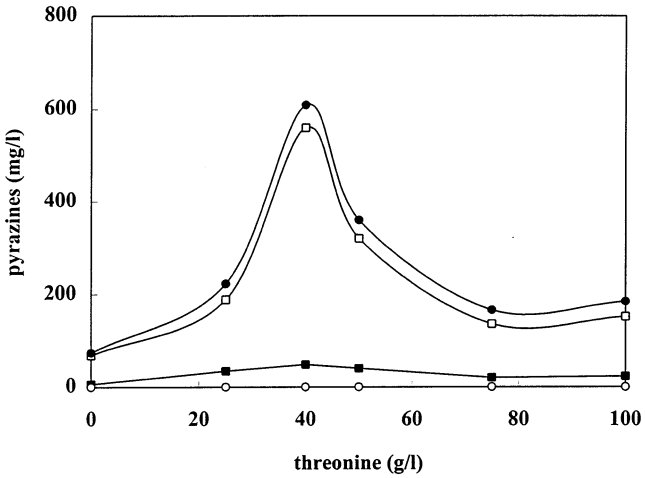

Fig. 5. Effect of L-threonine addition to the medium on pyrazine production. Total pyrazines, (●); 2,5-DMP, (□); TMP, (■); TTMP, (○). Data obtained after 15 days cultivation at 27°C in Erlenmeyers flasks.

2.4. Experimental design

Experiments were carried out either in Erlenmeyer flasks or in a bioreactor. In the first case, 500 ml flasks containing 200 ml of cultivation medium (see above) and fitted with a polyurethane foam plug (Caubere, Yebles, France) were placed on a rotating table and incubated at 27°C and 250 rpm.

The bioreactor used (SET 2, Inceltech, Toulouse, France) had a total volume of 2 l and contained 1 l cultivation medium. The aeration rate varied from 0.004 to 0.4 VVM, the stirring speed was 700 rpm, giving a volumetric oxygen transfer coefficient $k_L a$ of 180 h^{-1}, and the temperature varied from 27 to 40°C. Alternatively, the pH could be controlled at 7.5 by HCl (2.4 mol/l) or NaOH (5 mol/l) addition.

2.5. Assay methods

2.5.1. Measurement of viable cells

Viable cells in a sample where determined as colony forming units (CFU) obtained after growth for 20 h at 27°C of an appropriate dilution on Petri dishes. Each sample was treated in triplicate.

2.5.2. Volatile compounds

Pyrazines and acetoin where extracted by simultaneous steam distillation solvent extraction using a Likens and Nickerson apparatus [12] purchased from Chrompack (Middelburgh, The Netherlands). Sample (20 ml) was mixed with 5 μl 1-butanol used as internal standard and extracted for 2 h with 2 ml dichloromethane using a procedure already described [13].

The organic solution was directly analyzed by gas chromatography on a Supelcowax 10 (Supelco, Inc, Bellefonte, Pa, USA) capillary column as previously reported [9].

2.5.3. Non volatile compounds

The total sugar content in the whole medium was determined using the phenol/sulphuric acid colorimetric method of Dubois et al. [14], while total proteins were assayed, after solubilization by mixing 2 ml sample with 1 ml NaOH 3 mol/l and heating at 100°C for 5 min, using the Biuret method [15].

The other analysis were performed on the supernatant obtained after centrifugation of the whole medium at $12\,000 \times g$ for 10 min. These were soluble aminoacids, assayed using the ninhydrin-based colorimetric protocol of Lee and Takahashi [16] and ammonium ions, determined by means of the Berthelot colorimetric reaction [17].

2.5.4. Comparison of data from solid state and solid substrate cultivations

Results obtained during the course of the present work were expressed with respect to the volume of the slurry medium. Those achieved during solid state experiments were related to initial dry weight [9].

Comparison between these two processes was carried out by converting solid state data in order to express them in terms of volume of packed bed. This was achieved considering that 1 kg IDM corresponded to 4.24 l.

3. Results and discussion

3.1. Feasibility of solid-substrate fermentations for pyrazine production

The time course of cultivation parameters during a process carried out in an Erlenmeyer flask revealed the same periods as those evidenced in solid state cultivation [9]. Hence, three main phases appeared here also (Fig. 2). Active cell growth took place during the first 3 days, in connection with protein and sugar consumption. This time interval also corresponded to amino acids and ammonium liberation, resulting from proteolytic activity of *Bacillus subtilis* [9,18,19].

The major phenomenon occurring during the following 3 days (3–6 days) was a decrease in viable cell content in the medium. This feature could be attributed to autolysis, since *Bacillus subtilis* is known to synthesize autolysins, the major component being the *N*-acetylmuramoyl-L-alanine amidase that splits the linkage between the glycan and the peptide moiety of peptidoglycan in the cell wall [20]. This lysis does not occur during growth and indicates that conditions that

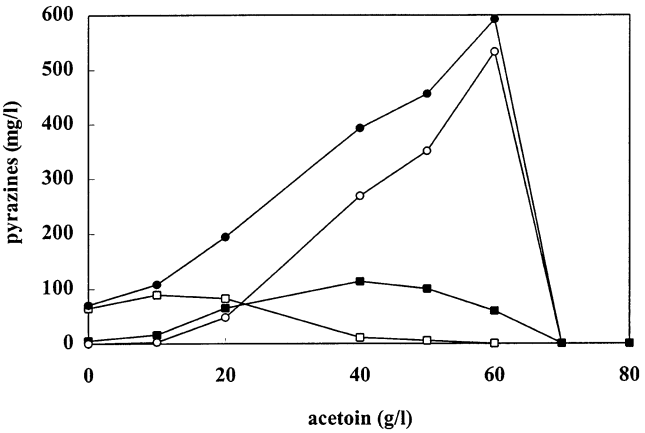

Fig. 6. Effect of acetoin addition to the medium on pyrazine synthesis observed after 15 days cultivation. Symbols and experimental conditions as in Fig. 5.

do not permit energy generation have arisen in the medium [20]. However, examination of the curves in Fig. 2 showed that neither carbon nor nitrogen sources were exhausted and the nature of the limitation could not be elucidated. The end of the cultivation was characterized by a stationary period without any significant change in growth parameters.

The three periods revealed during growth studies were also present when metabolite synthesis was examined (Fig. 3). The growth phase corresponded to acetoin accumulation, a phenomenon resulting from sugar catabolism and commonly observed with *Bacillus subtilis* [21]. It also corresponded to a medium acidification, that took place with organic acid production. Hence, 7 g/l isovaleric acid and 2 g/l isobutyric acid were detected after 3 days cultivation; these concentrations remained constant after this time (data not shown). These compounds resulted from leucine and isoleucine catabolism and have been reported to be produced in significant amounts during fermentation of soybeans [22,23].

The autolytic phase corresponded to medium alkalinisation and pyrazine synthesis (Fig. 3). Total pyrazine concentration reached 80 mg/l after 6 days cultivation, a time at which all metabolite formation processes stopped. The main aroma compound was 2,5-dimethylpyrazine (2,5-DMP), which accounted for 85% total pyrazines, followed by trimethylpyrazine (TMP, 10%), and tetramethylpyrazine (TTMP, 1.5%). The remaining consisted of minor amounts of 2-ethyl-5-methylpyrazine, 2-methylpyrazine, 2-ethylmethylpyrazine, and 2,3-dimethylpyrazine. It could thus be concluded that 2,5-DMP was the major product on standard solid substrate cultivation, as in standard solid state processes [9].

These promising results were obtained using Erlenmeyer flasks. The behaviour of the system in an aerated, stirred bioreator was then examined.

3.2. Use of an aerated, stirred bioreactor with standard ground soybeans: influence of aeration rate

The aeration rate had a dramatic influence on the behaviour of the system. Indeed, a very sharp optimum could be evidenced at 0.005 VVM for pyrazine synthesis (Fig. 4), corresponding to high oxygen limitation. These conditions allowed the recovery of about 200 mg/l total pyrazines, a value markedly higher than that obtained using Erlenmeyer flasks (80 mg/l).

The decrease in aroma compounds observed with increases in aeration rate could be due to loss of volatile components by air stripping, as in the case of methyl-ketones [24]. Experiments were thus carried out in this area with an aeration rate of 0.0084 VVM, a stirring rate of 700 rpm and at 27°C, either with distilled water or ground soybeans medium (pH = 6.5 or 8). Results demonstrated that the maximal loss did not exceed 4% after 12 days (data not shown). It was concluded that this phenomenon could be considered as negligible and that the curve in Fig. 4 reflected true pyrazine synthesis.

Table 1
Summary of the influence of L-threonine and acetoin addition to the cultivation medium on pyrazine production*

Precursor added[a]	2,5-DMP (mg/l)	TMP (mg/l)	TTMP (mg/l)	Total (mg/l)
None[b]	68	8	1	77
Acetoin[c]	0	61	530	591
L-Threonine[c]	560	49	1	610
Threonine + acetoin[c,d]	95	50	265	410
Threonine + acetoin[e]	560	66	437	1063

* Experiments carried out in Enlenmeyer flasks at 27°C using ground soybeans. Average relative error on the results was always less than 10%.
[a] L-Threonine and acetoin were added at concentration of 40 and 60 g/l, respectively.
[b] Values obtained after 6 days cultivation.
[c] Values obtained after 15 days cultivation.
[d] Simultaneous precursors addition at the beginning of the process.
[e] Threonine added to time 0, acetoin at 15 days, results obtained after 20 days cultivation.

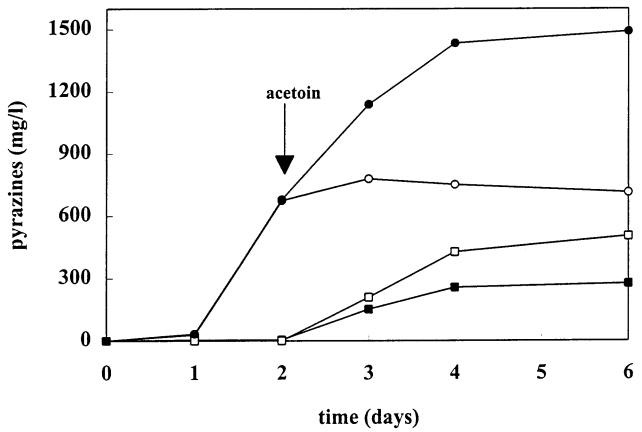

Fig. 7. Typical time-course of pyrazine synthesis at 40°C with sequential enrichment with threonine (40 g/l) and acetoin (60 g/l). Total pyrazines, (●); 2,5-DMP, (○); TMP, (■); TTMP, (□). Experiment carried out in bioreactor, stirring speed 700 rpm, aeration rate 0.04 vvm.

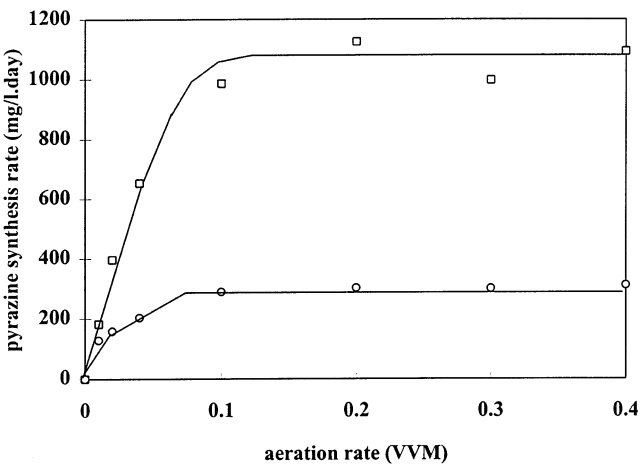

Fig. 8. Maximal 2,5-DMP (□) and TTMP (○) synthesis rates plotted against aeration rate. Experimental conditions, except for aeration, as in Fig. 7.

The sensitivity of *B. subtilis* to aeration conditions is a well-known phenomenon [25]. Pyrazine synthesis could then be seen as the expression of a metabolic overflow, maybe due to too low an efficiency of the respiratory chain in the microaerobic conditions required for this metabolism.

3.3. Optimisation of cultivation medium

3.3.1. L-Threonine addition

L-Threonine was previously found as being the precursor of 2,5-DMP for *B. subtilis* IFO 3013 [9]. Systematic studies, carried out in Erlenmeyer flasks, were performed in this area. Results obtained (Fig. 5) confirmed that soybeans enrichment with this amino acid could markedly improve pyrazine synthesis and 600 mg/l total pyrazines were produced when the medium was supplemented with 40 g/l threonine. The major compounds obtained in these conditions were 2,5-DMP (550 mg/l) and TMP (50 mg/l) while TTMP was virtually absent. These results were observed after 15 days cultivation at 27°C.

3.3.2. Acetoin enrichment

As expected [9], acetoin stimulate TTMP synthesis (Fig. 6). The optimum concentration was found to be 60 g acetoin/l, and TTMP accounted for c.a. 90% of total pyrazines.

As with solid state cultivations [9], this result was observed with a marked inhibition of 2,5-DMP production, since this aroma was no longer detected in the medium (Table 1). These result were also obtained after 15 days cultivation at 27°C.

3.3.3. Addition of threonine and acetoin

When both threonine and acetoin were introduced at the beginning of cultivation, each at its optimal concentration as determined above, a simultaneous increase in both 2,5-DMP and TTMP was achieved (Table 1). However, the synthesis of both pyrazines was lower than when each precursor was added separately. This phenomenon could be the result of acetoin inhibition of the bacterial metabolism with an early feeding of this compound, as when this compound was used alone.

This result led to experiments in which threonine was first introduced in to the medium, then acetoin was added after the end of 2,5-DMP production. This sequential approach led, as expected, to 2,5-DMP and TTMP concentrations close to those achieved when separate feedings were performed (Table 1). As a result, a strong increase in total aroma synthesis was observed, and total pyrazines concentration reached a value close to 1 g/l. This system was then used in subsequent experiments.

3.4. Further optimization using a bioreactor

3.4.1. Temperature

Experiments carried out by increasing the cultivation temperature revealed that a very strong increase in the aroma compound synthesis rate could be achieved in this way (results not shown). This feature was connected to an improvement in total aroma production, mainly due to 2,5-DMP and TMP concentrations increase in the medium.

The most suitable conditions were found to consist of cultivation carried out at 40°C, and further experiments were performed at this temperature. An example of pyrazine production in these conditions with sequential enrichment with threonine and acetoin is given in Fig. 7.

Table 2
Influence of the aeration rate on pyrazine production[a]

Aeration rate (VVM)	2,5-DMP (mg/l)	TMP (mg/l)	TTMP (mg/l)	Total pyrazines (mg/l)	Pyrazine synthesis stop (days)	
					2,5-DMP	TTMP
0.006	765	105	428	1298	7	10
0.01	545	298	349	1192	4	7
0.02	820	120	475	1415	3	6
0.04	692	271	503	1466	2	4.5
0.1	871	109	434	1414	2	3.50
0.2	655	269	533	1457	1.25	3
0.3	565	70	526	1161	1.25	3
0.4	513	250	542	1305	1.25	3
Average	678	187	474	1339		

[a] Cultures were performed at 40°C and 700 rpm in a bioreactor. Threonine (40 g/l) and acetoin (60 g/l) were added, respectively at 0 day and at the stop of 2,5-DMP synthesis.

3.4.2. Aeration

The cell response to aeration changes differed strongly from that observed on standard medium. The sharp optimum corresponding to microaerobic conditions disappeared when using enriched medium. Both 2,5-DMP and TTMP synthesis appeared oxygen limited for aeration rates lower than 0.1 VVM (Fig. 8). Higher values gave no change in the maximal aroma synthesis rates that remained close to 1 g/l day for 2,5-DMP and 0.3 g/l day for TTMP.

As a result, cultivation time was highly reduced and decreased from 10 to 3 days at 27 and 40°C, respectively. In all cases, the final pyrazine content in the medium was close to 1.3 g/l (Table 2).

Cell growth featured a behaviour close to aroma biosynthesis. Maximal viable cells appeared to depend on aeration rate in the oxygen limited region, and remained constant for values higher than 0.1 VVM (Fig. 9). This result could indicate a decrease in cell synthesis yield from the limiting (unidentified) growth substrate. It was also shown that the higher the maximal viable cell concentration, the higher the number of residual viable cells during TTMP synthesis.

It could thus be assumed that the metabolic state of growing cells differed from that of resting ones, the first category being responsible for 2,5-DMP synthesis while the latter produced mainly TTMP. This feature reflected the fact that biosynthetic pathways for these two compounds were different [26,27] (Fig. 10).

3.4.3. pH control

Cultivations took place with important changes in pH (see Fig. 3). Experiments were thus carried out in order to examine the effect of control of this parameter on aroma synthesis. Results showed that this strategy could give a further improvement in 2,5-DMP synthesis, while TTMP production remained almost unchanged. It was also noticed that branched-chain organic acids, isovaleric and isobutyric acids, that were synthesized when pH was not controlled, were no longer present when this parameter was stabilized.

This behaviour again emphasized the distinct metabolic pathways for synthesis of these two compounds. A final pyrazine content of 2 g/l was achieved in this way after 3 days cultivations (Table 3). It was the highest concentration obtained in this study.

4. Conclusion

Alkyl-pyrazine synthesis in *B. subtilis* appeared to be the expression of a metabolic overflow resulting from abnormal cultivation conditions, that could be achieved by two methods. Aroma overproduction could be observed by restricting oxygen availability if precursor

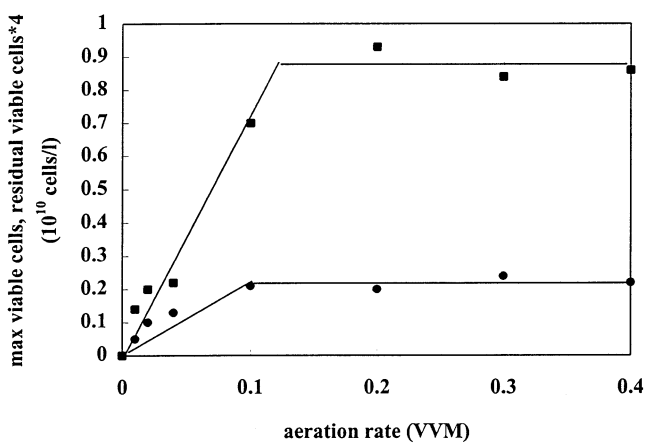

Fig. 9. Maximal (■) and residual (●) viable cells concentrations plotted against aeration rate. Residual viable cells are bacterial counts obtained during the stationary period. Experimental conditions as in Fig. 8.

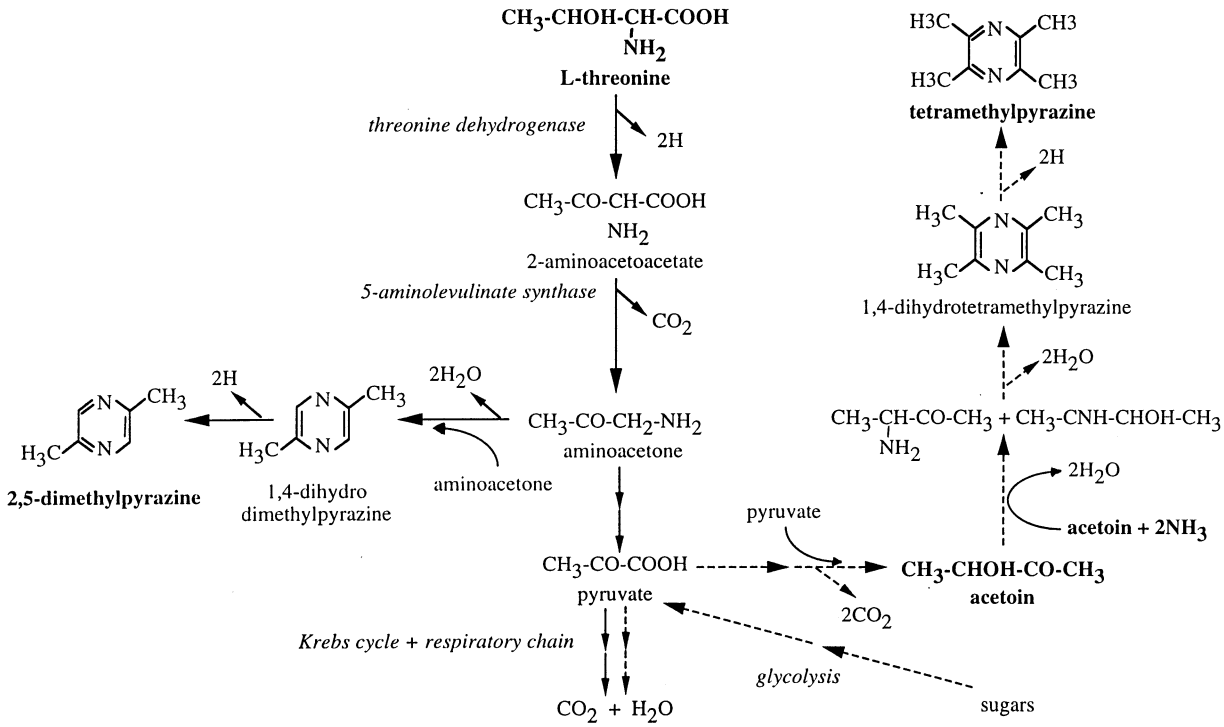

Fig. 10. Metabolic pathways for 2,5-DMP and TTMP synthesis. Resulting stoichiometric equations are as follows:

2 acetoin + Cof → TTMP + $4H_2O$ + $CofH_2$

2 threonine + 3Cof → 2,5-DMP + $2CO_2$ + $2H_2O$ + $3CofH_2$

where Cof and $CofH_2$ are an oxidized and reduced cofactor, respectively.

concentrations were low. The second method involved enrichment of the medium with L-threonine, for 2,5-dimethylpyrazine synthesis, and/or acetoin, for tetramethylpyrazine recovery. This latter approach was by far the most efficient in term of concentrations of aroma obtained. However, even the higher pyrazine content achieved revealed low precursor conversion yields, since the maximal 2,5-DMP synthesis yield from L-threonine did not exceed 0.0035 g/g while TTMP formation from acetoin took place with a 0.008 g/g yield (from data in Table 3), values to be compared to the corresponding stoichiometric ones, 0.91 g 2.5-DMP/g threonine and 0.77 g TTMP/g acetoin (from Fig. 10). It should also be noticed that other possible degradation products, with the exception of branched chain organic acids when pH was not controlled, that might result from amino acid catabolism were never found in the medium. The metabolism of the main precursors could be their total oxidation.

The highest values obtained in this study involved separate feeding of precursors and pH control. These two operations were possible in solid substrate cultivations because they allowed the use of submerged fermentations techniques. Solid state processes did not permit these procedures. However, comparison of results on the same basis (see Section 2) revealed that solid state cultivations remained competitive for tetramethylpyrazine production (Table 4).

The values obtained in this study were, at our knowledge, the highest obtained in the area of pyrazines production [28].

Acknowledgements

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Table 3
Influence of pH on pyrazine production in a bioreactor operated at 40°C with an aeration rate of 0.2 VVM, a stirring speed of 700 rpm and with sequential addition of threonine and acetoin[a]

pH	2.5-DMP (g/l)	TMP (g/l)	TTMP (g/l)	Total pyrazine (g/l)
No control	0.67	0.27	0.53	1.46
7.5	1.42	0.22	0.47	2.11

[a] Data are obtained after 3 days cultivation.

Table 4
Comparison of solid substrate and solid state cultivations for pyrazine production by *Bacillus subtilis* IFO 3013

Technique	2.5-DMP (g/l)	TMP (g/l)	TTMP (g/l)	Total pyrazine (g/l)
Solid state[a]	0.19[b]	0.05	0.003	0.24
	0.002[c]	0.05	0.58	0.63
Solid substrate[d]	1.42	0.22	0.47	2.11

[a] Data adapted from Besson et al. [9], considering that 1 kg initial dry matter corresponded to 4.24 l (see Section 2).
[b] Results obtained with threonine enrichment.
[c] Results obtained with acetoin enrichment.
[d] Data from Table 3.

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